Universidade de Aveiro

Departamento de Química

Ano 2014

MILENA BARBARA GROTH

Fotodegradação de estrona em matrizes aquosas, sequida por HPLC.

Photodegradation of estrone in aqueous matrix, followed by HPLC.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química Analítica e Qualidade, realizada sob a orientação científica do Doutor Valdemar Inocêncio Esteves, Professor Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Diana Luísa Duarte Lima, Investigadora de Pós-doutoramento do Departamento de Química da Universidade de Aveiro.

I dedicate this work to my family for their support.

o júri

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palavras-chave

Estrona, hormonas esteróides, desreguladores endócrinos, água, fotodegradação, substâncias húmicas, sequestradores de oxigénio singuleto, HPLC-FLD.

resumo

A importante colaboração de diversas personalidades ligadas à área da Desreguladores endócrinos (EDC) são compostos com efeitos negativos sobre a atividade endócrina das hormonas naturais em animais e humanos. Além dos produtos químicos industriais, alguns estrogénios (naturais e sintéticas) são considerados os EDCs mais potentes. A fotodegradação é uma importante via de remoção dos estrogénios do sistema aquático. A estrona (E1) é uma das hormonas esteróides mais abundante nos sistemas aquáticos.

Neste trabalho investigou-se a degradação de E1, sob radiação solar simulada, e a influência da presença de diferentes frações de HSs (HA, FA e XAD-4) de origem aquática no processo de fotodegradação. Foram ainda testadas águas naturais com diferentes teores de matéria orgânica (OM). Pesquisou-se, ainda a formação de oxigénio singuleto nas amostras irradiadas utilizando um sequestrador do mesmo..

Os resultados indicaram que E1 foi fotodegradado mesmo na ausência de OM com uma semi-vida de 6 h. A presença de HSs reforça a fotodegradação de E1 na presença de todas as fracções de HS a qual sofre aceleração com o aumento das suas concentrações. Foi investigada a influência das diferenças estruturais dos HA, FA e XAD-4 sobre a fotodegradação de E1. As experiências realizadas em águas naturais demonstraram o impacto da origem das amostras sobre a taxa de fotodegradação E1, a qual está relacionada com conteúdo de OM e com a salinidade. Para testar a formação de oxigénio singuleto durante a irradiação da matéria orgânica das águas naturais na presença de E1, foi utilizada a azida de sódio, como sequestrador de oxigénio singuleto. A presença de OM e azida de sódio, provaram reduzir a fotodegradação em ambas as amostras de águas residuais, mas o efeito foi mais pronunciado no caso da amostra de uma ETAR depois do tratamento primário (STPP). Assim, ficou provada a formação de oxigénio singuleto, quando se irradia a matéria orgânica, o qual intervem no processo de fotodegradação de E1.

As análises químicas de E1 foram feitas por HPLC-FLD tendo-se obtido um LOD de 17,4 µg/L.

keywords

Estrone, steroid hormones, endocrine disrupting compounds, water, photodegradation, humic substances, singlet oxygen scavengers, HPLC-FLD.

abstract

Endocrine disrupting chemicals (EDCs) are compounds with detrimental effects on the endocrine activity of the natural hormones in the human and animals. Besides industrial chemicals, some estrogens (both natural and synthetic) are found to be the most potent EDCs. Photodegradation is an important pathway for removing of estrogens from the aquatic system. Estrone (E1) is one of the steroidal estrogens existing widely in the aquatic environment and was found to be the most abundant estrogen in aquatic systems.

The main goal of this work was to investigate the photodegradation of E1 in water under simulated solar radiation and to estimate the impact of different fractions of HSs (HA, FA and XAD-4). So far not many studies conducted on the impact of OM, existing in natural water on the photodegradation of E1. Therefore, the same experiment were done in the natural water with different content of OM, depending on the origin. The testing of E1 photodegradation with addition of singlet oxygen scavenger have been also performed.

The results indicated that E1 was photodegraded even in the absence of OM with a half-life of 6 h. The presence of HSs enhanced the photodegradation in presence of all HS fractions accelerating the photodegradation of E1 with increasing concentrations of HSs. The effect of structural differences between HA, FA and XAD-4 on photodegradation of E1 has been studied. Experiments performed in natural water have shown the impact of origin of water samples on the rate of E1 photodegradation, which is related to different content of OM and salinity. The results of studies have shown inhibitory effect of the presence of sodium azide, as a singlet oxygen scavenger, on the photodegradation of E1. The presence of both OM and scavenger, have proven to reduce the photodegradation in both wastewater samples, but the effect was more pronounced in the case of sample after primary treatment (STPP).

The LOD of 17.4 μg/L was achieved with HPLC-FLD procedure for the analysis of E1 in aqueous samples.

Abbreviations:

DOM – Dissolved Organic Matter

E1 – Estrone

E2 - 17β-estradiol

E3 – Estriol

EDC – Endocrine Disrupting Compound

EE2 – Ethinylestradiol

ER – Estrogen Receptor

FA – Fulvic Acid

GC-MS – Gas Chromatography – Mass Spectrometry

HA – Humic Acid

HPIA – Hydrophilic Organic Acids

HPLC – High Performance Liquid Chromatography

HPLC-FLD – High Performance Liquid Chromatography with Fluorescence Detection

HPLC-UV - High Performance Liquid Chromatography with Ultraviolet Detection

HPOA – Hydrophobic Organic Acids

HS – Humic Substances

Hu – Humin

IHSS – International Humic Substances Society

LC-MS – Liquid Chromatography – Mass Spectrometry

LOD – Limit of Detection

MeEE2 – Mestranol

MS – Mass Spectrometry

MQ – Milli-Q water

OM – Organic Matter

POM – Particulate Organic Matter

STP – Sewage Treatment Plant

STPF – Final Effluent of STP

STPP – Primary Effluent of STP

TOC – Total Organic Carbon

UV – Ultraviolet

XAD-4 – styrene divinylbenzene

XAD-8 – poly(methylmetacrylate)

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Introduction

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1. Steroid hormones in the environment

Steroid hormones are a group of biologically active compounds containing a cyclopentan-o-perhydrophenanthrene ring, which are synthesized from cholesterol. Natural steroids include progestogens, mineralocorticoids, glucocorticoids, androgens, as well as estrogens (Raven and Johnson, 1999). The action of all steroid hormones involve their passing through the plasma membrane and binding to intracellular receptors. The predominant female natural hormones are estrone (E1), 17β-estradiol (E2), estriol (E3), which may influence the health of tissues, skin, breasts and brain. Besides natural steroids, there are some synthetic estrogenic steroids, such as mestranol (MeEE2) and ethinylestradiol (EE2), which are used in the formulation of contraceptive pills. The presence of estrogenic steroids in the environment arouses a great interest among the researchers, because they can disrupt the reproduction of humans, livestock and wildlife. Both humans and animals excrete steroid hormones, which reach the aquatic environment through sewage discharge and animal waste disposal, and can disrupt the functioning of hormonal system of humans and aquatic organisms (Jobling et al., 1998).

Sources of estrogenic compounds in the environment

In humans and other mammals, the natural and synthetic estrogens are metabolized in liver, where they are conjugated to glucuronide-, sulfate- or sulfoglucuronide- forms and then are excreted mainly via urine (Ternes et al., 1999a; Hanselman et al., 2003; Mao et al., 2004). The level of excreted steroids depends on age, diet, state of health and pregnancy (Lintelmann et al., 2003; Zheng et al., 2008; Johnson et al., 2000). The studies of excretion rates of natural estrogens by women determined is in the lower microgram range of excretion. The estrogen excretion detected for women at a reproductive age (15-59 years) varied from 5-31 μg/day (E1), 3-19 μg/day (E2) and 4-33 μg/day (E3). Moreover the highest level of excretion of estrogen was found for pregnant women in the values of 600-940, 170-330 and 4.500-6.500 μg/day for E1, E2 and E3, respectively (Johnson et al., 2000; D'Ascenzo et al., 2003; Johnson and Williams, 2004). In the case of man, the estrogen excretion level is about 4-12, 1.5-7 and 1.5-6 μg/day for E1, E2 and E3, respectively (Johnson et al., 2000; D'Ascenzo et al., 2003; Johnson and Williams, 2004).

Table 1 shows the estimated daily excretion of estrogens of males and females, based on the survey of human estrogen excretion obtained by Johnson et al. (2000).

Table 1. *Daily excretion (μg) of estrogenic steroids in humans (Johnson et al., 2000).*

Category	**E2**	**E1**	**E3**	**EE2**
Males	1.6	3.9	1.5	-
Menstruating females	3.5	8.0	4.8	-
Menopausal females	2.3	4.0	1.0	-
Pregnant women	259	600	6000	-
Women	-	-	-	35

Humans generally produce natural estrogens in the microgram range per day, while concentrations of these compounds in municipal wastewater and surface water receiving effluent is found in the nanogram range per liter (Ternes et al., 1999; Baronti et al., 2000; Yoshimoto et al., 2004; Zorita et al., 2009). These hormones are not degraded during the wastewater treatment process and are released to the environment with the effluent. Therefore, wastewater treatment processes are the main source of estrogens in aquatic ecosystems, because of the incomplete removal of these compounds (Gomes et al., 2003; Leech et al., 2009; Liu et al., 2009).

Some hormone steroids can come from livestock waste, such as, from sheep, pigs, poultry and other animals, which excrete steroids such as E1, E3 and E2 in two different epimer forms (Sarmah et al., 2006). The daily estrogens excretion depends on the sex, age, animal species, circadian cycle and reproductive state (Lange et al., 2002; Shore and Shemesh, 2003). Several studies have been performed with poultry waste and an average of 44 ng/g for E2 have been reported (Shore et al., 1988; Shemesh and Shore, 1994). It was proven that disposal of animal manure to agricultural land is the reason of the presence of estrogenic steroids in surface and ground water (Nichols et al., 1997; Bushée et al., 1998; Peterson et al., 2001). In addition, the use of steroid drugs as growth promoters in livestock was also reported (Schiffer et al., 2001). They are frequently used, e.g. in cattle livestock, in order to treat reproductive disorders, control the oestrous cycle and induce abortion (Refsdal, 2000). The addition of steroid drugs can greatly increase the production of hormone steroids in urine of livestock (Callantine et al., 1961). It has been assessed that

about 2.7 mg/L in urine per person on a daily basis is one of the main sources of steroid compounds in the aquatic environment, while the excretion of these steroid hormones by animals is limited to livestock waste from the animal industry (Kolpin et al., 2002; Zuo et al., 2006).

Occurrence of steroids in aquatic environment

The steroid hormones, contained in domestic sewage and livestock wastewater, are directly discharged to the rivers or with the effluent of sewage treatment plants (STPs) or through runoff of sewage sludge, reach to the aquatic environment (Hanselman et al., 2003; Samir et al., 2006). Numerous studies have demonstrated the presence of endocrine disrupting compounds, especially estrogenic compounds, in wastewater and natural waters (Desbrow et al., 1998; Ternes et al., 1999a; Kuch and Ballschmitter, 2001; Lin and Reinhard, 2005; Racz and Goel, 2010). The natural and synthetic estrogens have been detected in both influents and effluents of STPs and their concentrations generally ranged from 10 to 200 ng/L (Ternes et al., 1999a; Baronti et al., 2000; Andersen et al., 2003; Labadie and Budzinski, 2005; Zhang and Zhou, 2008). The natural estrogens (E1 and E2) and synthetic one (EE2) were detected as a major estrogenic compounds in urban wastewater effluents (Desbrow et al., 1998).

In the literature, there are several reports about the levels of steroids in surface waters (Belfroid et al., 1999; Baronti et al., 2000; Kuch and Ballschmiter, 2001; Duong et al., 2010). The concentrations of estrogenic compounds have been detected in the range of ng/L, but high concentrations (up to μg/L) have been also detected (Kolpin et al., 2002; Hohenblum et al., 2004). E1 was found in estuarine and freshwater samples with an average concentration of 0.3 ng/L, while the concentrations of E2 and EE2 was below the quantification limit of < 1.0 ng/L (Belfroid et al., 1999). Estrogenic steroids were also found in some drinking water samples in Germany (Kuch and Ballschmiter, 2001) and river water in Italy, where the concentration of E1, E2 and E3 was 1.5, 0.11 and 0.33 ng/L, respectively (Baronti et al., 2000). The high level of estrogens in polluted streams and rivers, with the concentrations of 112 ng/L for E1, 200 ng/L for E2 and 51 ng/L for E3, have been detected (Kolpin et al., 2002). The presence of hormone steroids in ground water have been also proven by several researchers (Shore et al., 1995a; Peterson et al.,

2001). E2 and E1 were detected in only 1/5 of the groundwater samples, but high level of steroids in samples close to urban areas have been reported (Hohenblum et al., 2004).

The effect of estrogenic compounds to the environment

Traditional methods of water and sewage treatment are not completely effective in removing estrogens therefore the knowledge about their effect to the environment is very important (Coleman et al., 2000). Estrogenic steroids belong to the group of compounds, which can interact with the endocrine system of humans and animals, leading to interfere with the normal function of hormones (Jobling et al., 1998). These compounds, called "endocrine disrupting compounds" (EDCs), represent a potential threat to human health and aquatic life (Zhang and Zhou, 2005; Zuo et al., 2006) and arouse great interest among researchers since the early 1990s, because their effect on ecosystem and human health is not yet completely known.

Their negative effect on the endocrine system of humans and animals, consist of mimicking and antagonizing the effect of endogenous hormones, disturbing the production of specific hormone receptors or disrupting the synthesis and metabolism of endogenous hormones (Stoker et al., 2000; Mendes, 2002; Caserta et al., 2008; Matozzo et al., 2008; Roy et al., 2009). Health effects caused by the presence of estrogenic steroids, which have been demonstrated on humans include altered sex ratios (Mocarelli et al., 1996), increase in the incidence of female breast cancer (Wolff and Toniolo, 1995), undescended testicles (Toppari et al., 1996), the decrease in the numbers of spermatozoa (Carlen et al., 1992) and neurological effects (Brody and Rudel, 2003). They imitate estrogens and bind to estrogen receptors (ERs) in the human body, resulting in the changes in the homeostasis of the endocrine system (Roy et al., 2009).

The presence of estrogens, particularly of E1, E2, EE2 and E3, even at such low concentrations as ng/L, may cause serious risks, due to their high estrogenic activity (Conroy et al., 2007; Racz and Goel, 2010). These estrogenic compounds may cause synthesis of female specific protein (vitellogenin) and feminization in male fish, which have been observed in British rivers (Desbrow et al., 1998; Jobling et al., 1998). Their impact on the sexuality, and reproduction of both invertebrates and vertebrates has been proven (Kang et al., 2002; Brion et al., 2004). Due to the strong affinity of estrogens for

ERs, even low estrogen concentrations can cause endocrine disruption in a wide range of wildlife populations (Combalbert and Hernandez-Raquet, 2010). The negative impacts include feminisation of males (Rodger-Gray et al., 2001), masculisation of females (Matthiessen and Gibbs, 1998) and reproductive abnormalities (Lye et al., 1997).

Besides of the impact of the presence of hormone steroids on humans and wildlife, they can affect also plants (Shore et al., 1995b; Lim et. al., 2000). Irrigation with sewage effluent containing hormone steroids have caused increasing of levels of phytoestrogens (Shore et al., 1995b). For these reasons removal of estrogens is very important in order to avoid the potential risks caused by the presence of estrogens in aquatic environments (Silva et al., 2012; Ying et al., 2002).

The steroid hormones have high biological potency and procreation toxicity, despite their concentration in natural aquatic environments is in the range of nanogram per liter (10-1830 ng/L), therefore it is important to understand their fate in the environment (Desbrow et al., 1998; Brion et al., 2004). Typical representatives of EDCs, including the natural and synthetic hormones are shown in the Figure 1.

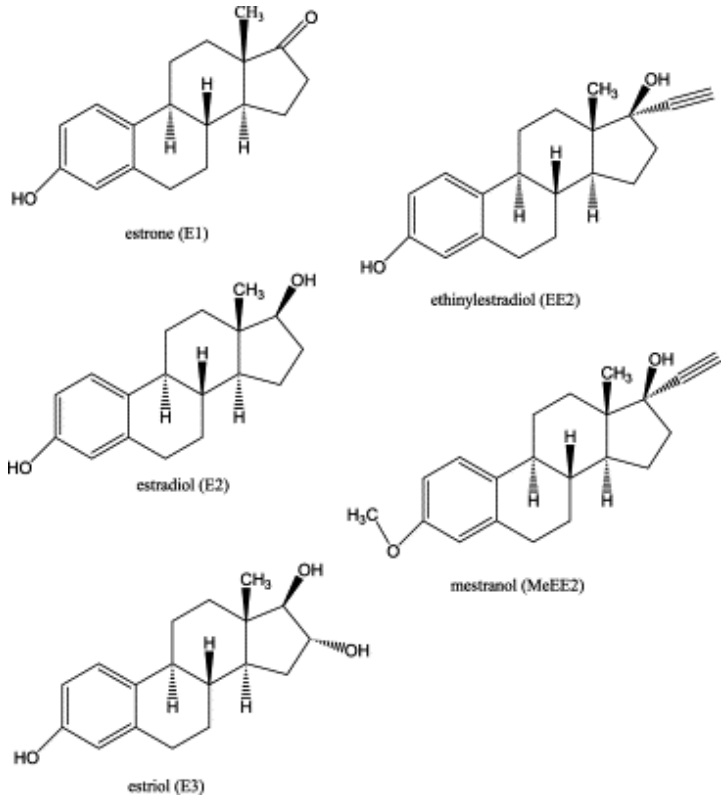


Figure 1. *Structures of hormone steroids (Ying et al., 2002).*

Degradation of estrogens in the natural environment

The behavior and decomposition of estrogenic steroids, such as E1, E2, E3 and EE2 depend on their physicochemical properties and the environmental characteristics. Both natural and synthetic estrogens contain substances with a high pharmacological activity and a certain amount of them enter the aquatic environment. In order to avoid the risks associated with the presence of estrogens in water, they should be eliminated before final release into the environment. Elimination of these steroid hormones can be performed by using physical processes, such as sorption, biological processes (biodegradation) or oxidation processes (photolysis and photocatalysis).

Sorption elimination involves the uptake of hormones from the aqueous phase on a solid phase (sorbent). This approach is proved to be efficient, effective and economic purification method, in which different adsorbent can be used (Qu, 2008). Because of the properties of estrogen compounds (poorly soluble in water, low biodegradability, hydrophobicity and low Henry's Law coefficients), they can easily adsorb onto solids in the wastewater activated sludge process (Ren et al., 2007), so use of adsorbent materials can be an efficient way to their removal from aquatic environment. Sorption ability may be related to sorbent's characteristics, such as particle size, pH, organic carbon content, salinity and ion content (Clara et al., 2004; Lei et al., 2009). It was proven that free unconjugated estrogens are more likely to sorb onto sludge, because of their greater hydrophobic properties (Janex-Habibi et al., 2009). The difference between sorption affinity of natural and synthetic estrogens were studied (Ren et al., 2007) and more readily removal of synthetic estrogens from the water phase has been proven (Lai et al., 2000).

Biodegradation is the primary removal method for estrogens in wastewater, in which microorganisms naturally occurring in the environment plays the most important role and can transfer or modify the structure of chemicals, introduced to the environment. This process includes deconjugation, degradation for heterotrophic biomass, cometabolism with nitrifying biomass and other cometabolism (Yoshimoto et al., 2004; Braga et al., 2005; Stumpe and Marschner, 2009). The studies demonstrated greater abundance the unconjugated forms of estrogens in wastewater effluents, than the conjugated forms, although estrogenic steroid hormones are mainly excreted as inactive conjugates of sulphuric and glucuronic acids (Ying et al., 2002). This is a result of transformation the

excreted inactive conjugates back to the active unconjugated forms by microorganisms (Racz and Goel, 2010). Some organic species are degraded by organisms, which utilise these compounds for growth. This method is responsible for the biodegradation of the natural estrogens (Vader et al., 2000). Another important biodegradation process, in which estrogens are modified but are not utilised for growth, is called cometabolism (Alexander, 1994; Vader et al., 2000). The degradation of organic compounds depends on their bioavailability and also on the ability of microbial organisms to transform and degrade them (Stumpe and Marschner, 2009). The effect of initial concentration of estrogens was also studied and increasing degradation rates with increase initial concentration of estrogens has been proven (Ke et al., 2007). Moreover, the greater concentration of total organic carbon (TOC) caused more difficult degradation of estrogens, because bacteria begin to utilize other organic compounds before using estrogens (Urase and Kikuta, 2005).

Photodegradation is one of the significant methods for removing estrogens from the aquatic environments, which can occur by exposing them to sunlight or by using photocatalysts. Natural sunlight can degrade all estrogens to some degree in both river water (Lin and Reinhard, 2005) and seawater (Zuo et al., 2006). Chromophoric moieties present in the structure of estrogens can absorb ultraviolet (UV) beam from solar radiation. During the release of estrogens into the environment, they are exposed to solar radiation, including both the UVA and UVB wave band of the solar spectrum, with the wavelength in the range of 320-400 nm and 280-320 nm, respectively. For this reason the investigation of estrogen behavior under UV radiation is important. Degradation in the aquatic environment may occur via two principal processes: direct and indirect photolysis. In the direct photolysis, light is absorbed directly by the chemical itself, leading to bond cleavage (Liu and Liu, 2004; Rosenfeldt and Linden, 2004). Indirect photolysis consists of light absorption by photosensitizers which generate many photoreactants, such as solvated electrons, OH radicals (·OH), singlet oxygen ($^1O_2$) or superoxide (Lin and Reinhard, 2005). The next step involves the reaction of these photoreactants with the compounds of interest. The most important photosensitizers are natural dissolved organic matter (DOM), nitrate and nitrite (Lin and Reinhard, 2005; Atkinson et al., 2011). Indirect photolysis can play an important role on the degradation of pollutants that resist to direct photolysis (Zafiriou et al., 1984; Boule et al., 2005). Some compounds may subject to both direct and indirect photodegradation in aqueous solution, as was proved in the case of E3 (Chen et al., 2013).

The other method, which proved to be an effective method for removal of estrogens from aquatic environment, is heterogeneous photocatalysis (Augugliaro et al., 2006), which involves the use of catalysts, such as titanium dioxide (TiO_2), ferric oxide (Fe_2O_3), zinc oxide (ZnO) and silicon (Si) (Augugliaro et al., 2006). The most widely used photocatalyst is TiO_2, because of its high stability, non-environmental impact, considerable activity and low cost (Augugliaro et al., 2006). The effectiveness of the heterogeneous photocatalysis using TiO_2 was demonstrated by several researchers (Coleman et al., 2004; Zhang et al., 2007; Zhang and Zhou, 2008). Tanizaki et al. (2002) studied the catalytic photodegradation of estrogenic compounds by using TiO_2 photo-semiconductor thin films under UV light and obtained decomposition rate of E1, E2 and EE2 as 0.058, 0.015 and 0.050 min^{-1}, respectively. The study demonstrated that only 20% decomposition of initial concentration of E1 were obtained by direct UV irradiation, but TiO2 photocatalytic oxidation caused the 90% its decomposition after 30 min of irradiation (Tanizaki et al., 2002). Moreover, removal of estrogenic activity of estrogens was more effective using photocatalysis over TiO_2 than direct photolysis (Coleman et al., 2004). The studies of estrogens degradation by using UV irradiation coupled with TiO_2 or Fe (III) and H_2O_2 demonstrated the removing of 98% of E1, E2 and EE2 (Feng et al., 2005; Benotti et al., 2009).

2. *Effect of organic matter on the degradation of estrogens*

Organic matter (OM) is found in dissolved or particulate forms in ocean and fresh water. In all aquatic samples (even in a clear water, such as that from the open ocean), there is at least small fraction of OM with the typical concentrations in the range of 1-3 mg/L. The OM is divided into two broaden classes – particulate organic matter (POM) and dissolved organic matter (DOM) (van Loon and Duffy, 2000).

Presence, isolation and properties of HSs occurring in natural water

Humic substances (HSs) are complex organic compounds, which result in yellow-brown colour of swamp or bogs, as well as to a lesser extent, of some lakes and rivers (Akkanen, 2002). HSs are located in water and soil, forming a major component of both aquatic (dissolved organic matter, DOM) and terrestrial (soil organic matter), carbon pools. According to the knowledge HSs are formed from the decay of bio-matter and are the part of DOM with high resistance of microbial degradation (Akkanen, 2002). In the surface water from boreal region, up to 90% of DOM is believed to be HS, though the global average value of HS corresponds about 50% of the total DOM (Akkanen, 2002). The rest of DOM, not acting the HSs, are mainly amino acids, carbohydrates, low molecular weight organic acids and fatty acids and is called the labile fraction of DOM (Akkanen, 2002).

HSs present in soil are different from aquatic HSs, due to their structural and chemical compositions (Tumdedo, 2010). Functional groups occurring in the structure of HS are responsible for its UV absorption, chemical binding, charge and hydrophobic interactions. The principal functional groups in HS are phenolic, hydroxyl, carboxyl and carbonyl (Thurman, 1985). HSs often are subdivided into subclasses according to their solubility in different media (van Loon and Duffy, 2000):

- Fulvic acid (FA) – humic substances' fraction soluble in aqueous solutions in all pH values
- Humic acid (HA) – humic substances' fraction insoluble under acid conditions (pH=2), but soluble at raised pH values
- Humin (Hu) – insoluble in water in all pH values.

The most common procedure using to isolate of HSs from freshwater is the XAD resins procedure, proposed by International Humic Substances Society (IHSS), which is based on the adsorption of compounds on XAD-8 (poly{methylmetacrylate}) and XAD-4 (styrene divinylbenzene) resins (http://www.humicsubstances.org). The use of these two extraction columns in tandem allows to extract both hydrophobic organic acids (HPOA) and hydrophilic organic acids (HPIA). Pre-filtered water samples are acidified to pH 2, by using hydrochloric acid (HCl) and passed through the resins (Stubbins et al., 2008). Acidification of samples allows protonation of carboxyl groups in HSs, which result in their sorption onto the hydrophobic resins (Mopper et al., 2007). XAD-8 resin has a great affinity for HPOA, therefore they are adsorbed and eluted from the column, using a strong base, such as sodium hydroxide solution (NaOH), (Stubbins et al., 2008). The obtained HPOA are further separated into HA and FA, by acidifying the eluate using HCl to pH<1 and desalted (Malcolm, 1991; Stubbins et al., 2008). The use of XAD-4 resin after XAD-8 allows to isolate HPIA (Stubbins et al., 2008), constituting the so called XAD-4 fraction (Mopper et al., 2007; Esteves et al., 2009).

Based on the hydrophobic and hydrophilic character of HSs, isolated on XAD suite of resins, they are split into the fractions (Braun et al., 2004; Mopper et al., 2007):

- Hydrophobic organic fractions adsorbed at low pH values (pH ≤ 2) onto XAD-8 resin and desorbed at high pH values (pH > 12), followed by:
    - precipitation at low pH to obtain HA;
    - desalting step with cation exchange to obtain FA;
- Hydrophilic organic fraction, which is not adsorbable at low pH values and is isolated by XAD-4 resin to obtain so called XAD-4.

The analytic studies of HPIA and HPOA, isolated from the same DOM sample, demonstrated lower % aromaticity, greater oxygen content, greater % alkyl and greater concentration of carboxyl for HPIA (Ma et al., 2001; Maurice et al., 2002).
The main properties of the three fractions of HS that were used in this work to study the effect of HS on the photodegradation, are presented in the Table 2.

Table. 2. *Main properties of aquatic HSs.*

(Filho and Silva, 2012; Santos, 1994; Esteves, 1995).

CONTENT OF COMPONENTS	Carbon	XAD-4 < HA and FA
	Oxygen	HA < FA < XAD-4
	Nitrogen	> XAD-4 and HA < FA
	Protonated organic carbon	XAD-4 < FA < HA
	Aromatic carbon substituted with oxygen	XAD-4 < FA < HA
	Methoxyl groups	XAD-4 < FA < HA
MOLECULAR WEIGHT		FA 500 – 2 000 units HA 20 000 – 100 000 units
CHEMICAL FORMULA		FA $C_{135}H_{182}O_{95}N_5S_2$ HA $C_{187}H_{186}O_{89}N_9S_2$
HYDROPHOBICITY		XAD-4 ≈ FA < HA

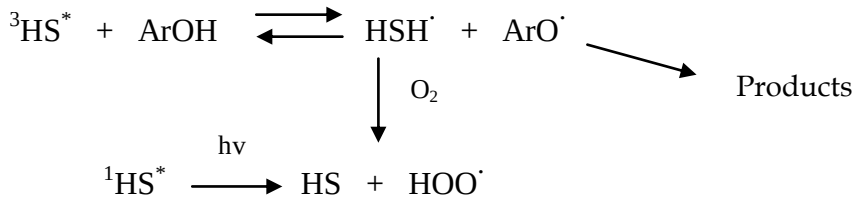
Impact of HSs on the photodegradation

Natural DOM is present in aquatic environment and can initiate a large number of photochemical reactions (Boule et al., 2005), caused by action of the light. The substances which, through absorption of light, are able to induce a chemical reaction in another compound are called photosensitizers (Canonica et al., 2009). HS are an example of this kind of substances. HSs contain chromophoric structures which are sensitive to UV-Vis sunlight and can absorb some part of solar radiation that reaches to aquatic system (Wetzel et al., 1995). The consequence of light absorption is a significant modification of structural and optical properties of HS (Osburn et al., 2001; Carvalho et al., 2008).

Sunlight irradiation of natural waters causes a transition of HS to an excited state, by absorption of the solar radiation (Aguer et al., 1999), according to reaction (1):

$$HS \xrightarrow{hv} {}^1HS^* \longrightarrow {}^3HS^* \qquad (1)$$

In the excited triplet states of OM, there are two unpaired electrons with three possible spin configurations, which cause the high reactivity of these molecules. HS, as a photosensitizer in the triplet state (^{3}HS*) can react in two ways. In the first way, ^{3}HS* reacts firstly with the substrate, causing the production of free radicals. During this pathway, electrons or hydrogen atoms are transferred between the excited state of the photosensitizer and the substrate (Aguer et al., 1999; Bancirova, 2011). The reaction (2) shows the first possible reaction pathway of $^3HS^*$.



(2)

In the second pathway, $^3HS^*$ reacts firstly with molecular oxygen leading to the production of singlet oxygen, in accordance with reaction (3). This process involves energy transfer between triplet excited states of HS and oxygen in the ground state (Li et al., 2010, Bancirova, 2011; Chen et al., 2013).

$$^3HS^* + {}^3O_2 \longrightarrow {}^1HS^* + {}^1O_2 \quad (3)$$

$$^3HS^* + {}^3O_2 \longrightarrow HS^+ + O_2^- \quad (4)$$

Excited triplet state of HS has been proposed to be transient oxidants for organic substrates and can induce their oxidation by hydrogen or electron atom transfer (Canonica et al., 2008). After absorption of light, HS can generate a high number of reactive species, such as singlet oxygen, superoxide, hydroxyl and peroxyl radicals, solvated electron and triplet excited state, which are highly reactive and indirectly cause photodegradation of organic compounds (Zepp et al., 1985; Carvalho et al., 2008; Guerard et al., 2009). Enhancement of photodegradation of estrogens, by HS acting as a photosensitizer (5), has been proven by several researchers (Leech et al., 2009; Chowdhury et al., 2010; Caupos et al., 2011; Grebel et al., 2012; Chen et al., 2013).

$$E1 + {}^1O_2 / \cdot OH \longrightarrow \text{products} \quad (5)$$

Apart from enhancing effect of HS on the photodegradation, it can also hamper the degradation of estrogens, acting as an absorber of light, which cause reduce quantity of photons available for photoreactions. It was also proven that HS may acts as a free radical quencher: hydroxyl radicals (Brezonik and Fulkerson-Brekken, 1998) or carbonate radicals (Canonica et al., 2005) to inhibit the degradation (6).

$$HS/\ {}^{1}HS^{*}\ /\ {}^{3}HS^{*}\ /\ HS^{+} + \cdot OH \longrightarrow \text{Oxidized HS} + H_2O \qquad (6)$$

The study of HA' impact on the photodegradation of E3 showed enhancement of degradation, when HA was working as a photosensitizer, as well as inhibitory effect of HA, acting as a radical quencher and light screening agent, has been proven. Total effect depends on the competition between these two roles (Chen et al., 2013). In the presence of HA, use of high intensity of light has caused production of more photoactivated HA species, which are more efficient free radical scavengers, resulting in the inhibition of the E3 photodegradation (Chen et al., 2013).

Nevertheless, the effect of HS on the photodegradation of organic compounds is not easy to explain. On one hand HS may induce the photodegradation of organic species by production of reactive intermediates (Guerard et al., 2009; Leech et al., 2009; Chowdhury et al., 2010; Caupos et al., 2011), but on the other hand they can act as inhibitors of photodegradation by absorption of the available photons or by the hydrophobic interaction between HS and organic compounds in solution (Bachman and Patterson, 1999; Canonica and Laubscher, 2008; Atkinson et al., 2011; Grzybowski and Szydłowski, 2014). Additionally, it has been investigated that the aromatic compounds can bind to the HS forming complexes, which are non-reactive under solar radiation and are not degraded (Rav-Acha and Rebhun, 1992). It was found that the inhibition or enhancement role of HS in the photodegradation depend on the type and quality of DOM (Atkinson et al., 2011), as well as on the incident light intensity (Chen et al., 2013). Generally, inhibitory effect of HS was observed with high incident light intensity, while acceleration of degradation has been proven under weak irradiation, such as sunlight (Chen et al., 2013).

3. Impact of singlet oxygen scavenger on the degradation of estrogens

Under solar radiation, natural waters produce highly reactive species, such as hydroxyl radical ($HO^{\cdot}$) and singlet oxygen ($^1O_2$) (al Housari et al., 2010). The last mentioned here, is a higher energy state of molecular oxygen (O_2) species and is considered highly potent oxidant with short half-life, which takes part in various biochemical and chemical reactions. $^1O_2$ is non-radical derivative of oxygen, which is formed by a spin reversal of electron in outer orbital of oxygen molecule (Tandon et al., 2005). Many photochemical reactions with the participation of oxygen have been studied and the molecular oxygen as a source of $^1O_2$, in the presence of the photosensitizer and visible light, have been proven (Bancirova, 2011). HSs are known as sensitizers for the $^1O_2$ production (Baxter et al., 1982; Braun et al., 1986; Hessler et al., 1996). The mechanism of $^1O_2$ formation involves the energy transfer between triplet excited states of HS ($^3HS^*$) and the ground state of O_2 (Aguer et al., 1999; Cory et al., 2009), as follow:

$$^3HS^* + {}^3O_2 \longrightarrow HS + {}^1O_2 \qquad (7)$$

The quantum yield of the production of $^1O_2$ by HSs presented in natural waters depends on the origin of HS from natural water (Braun et al., 1986; Haag et al., 1984). It was proven, that about 1-3% of the photon rate absorbed by HS leads to the $^1O_2$ production (Frimmel et al., 1987). $^1O_2$ is highly reactive and its reactions very often involve carbon-carbon double bond (Ameta et al., 1990; Aguer et al., 1999), which are present in many estrogens, such as E1, affecting to rate of its photodegradation.

It is proven that scavengers can inhibit reaction dependent of $^1O_2$ (Wilkinson and Brummer, 1981), which resulted in the interest of researchers to study effect of scavengers on the photodegradation of E1 (Caupos et al., 2011). The quenching of $^1O_2$ involves the deactivation of the excited state of molecule, which can be realized by both chemical and physical quenching. During chemical quenching $^1O_2$ reacts with quencher, by contrast to physical quenching, which consist in deactivation of $^1O_2$ to its ground state with no oxygen consumption and product formation (Halliwell and Gutteridge, 1989). The singlet oxygen scavengers involve physical scavengers (for example sodium azide, NaN_3) and chemical scavengers (carotene, ascorbate, thiols). In present work, NaN_3, as a physical scavenger was used to study the effect of singlet oxygen scavenger on the photodegradation of E1.

In fact, NaN_3 is mainly known as a physical quencher of $^1O_2$, but it has to be mentioned, that it is also able to react with $HO^{\cdot}$, forming the azidyl radical (Bottu, 1989; Halliwell and Gutteridge, 1990) and quench the excited states of some photosensitizers (Bensasson et al., 1993). The kinetics of the photolysis of NaN_3 was investigated by Peiris and Russell (2003) and the photolytic mechanism was proposed, as follows:

$$N_3^- + h\nu \longrightarrow N_3^{\cdot} + e^- \quad (8)$$

$$Na^+ + e^- \longrightarrow Na \quad (9)$$

The $N_3^{\cdot}$ radical appears to be very strong one-electron oxidant (Bancirova, 2011), which can react via any of three ways (Peiris and Russell, 2003):

$$N_3^{\cdot} + e^- \longrightarrow N_3^- \quad (10)$$

$$N_3^{\cdot} \longrightarrow N^{\cdot} + N_2 \quad (11)$$

$$N_3^{\cdot} + N_3^- \longrightarrow N_6^{\cdot -} \quad (12)$$

The scavenging action of NaN_3 involves the reaction with $^1O_2$ to form a reactive azide radical, as is shown in the following reaction (al Housari et al., 2010):

$$N_3^- + {}^1O_2 \longrightarrow N_3^{\cdot} + O_2 \quad (13)$$

In present work, the study of azide scavenging effect was performed in purified water and in water samples: STPP and STPF, where OM naturally occur in large quantities.

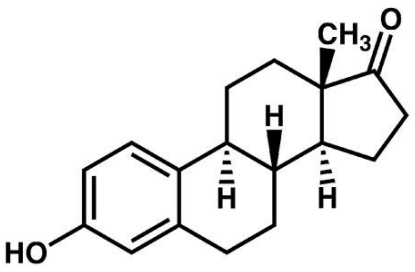
4. Estrone (E1)

Structure, formation and properties

Estrone (E1) is C_{18} steroid, constituted by 18 carbon atoms, which form three hexagonal and one pentagonal rings and include hydroxyl and carbonyl group. E1, as other estrogens, can be derived from cholesterol and pregnenolone, which are transformed to testosterone and androstenedione. These compounds are the precursors of the E1, as well as for the other estrogens (Combalbert and Hernandez-Raquet, 2010). Testosteron can be easily formed by reduction of androstenedione, therefore the major precursor of E1 synthesis is androstenedione. The formation of E1 is possible by 2 ways: directly from androstenedione (by three-step aromatization) or by the degradation of E2 (Renwick and Engel, 1967; Lee and Liu, 2002; Combalbert and Hernandez-Raquet, 2010).

The physicochemical properties of compounds determine their behavior and fate in the environment. Compounds with high K_{ow} values (higher than 10^4) have hydrophobic nature and high sorption potential (Jones-Leep and Stevens, 2007).

Table 3. *Physicochemical properties of estrone (Lai et al., 2000).*

	Estrone (E1)
Structure	
Molecular weight (g/mol)	270.4
Water solubility (mg/l) at 20°C	13
Log K_{ow}	3.43
Vapour pressure (mm Hg)	$2.3 \cdot 10^{-10}$
Relative estrogenic activity	2.54 [a]

[a] Pillon et al., 2005 (MELN in vitro test)

The physicochemical properties, such as low vapour pressure, high K_{ow} value and low water solubility show that E1 is an organic compound of low volatility, hydrophobic nature and high sorption potential. It can be expected that the sorption on soil or sediment will be an important factor in reducing its aqueous phase concentrations (Lai et al., 2000). Estrogens can exist in free forms or conjugated by glucuronide or sulfate, which cause their different properties and biological activity. Conjugated forms of E1 don't have biological activity and dissolve in water at a larger quantity comparing with its unconjugated forms (Khanal et al., 2006).

E1 is one of the three natural estrogens, besides E2 and E3, occurring in women. This is a biologically active steroid hormone, which is naturally excreted by women in the daily range of 2-12 μg/person, female animals and men (5 μg/person), (Belfroid et al., 1999). It was proven that during late pregnancy, E1 is excreted in high levels mainly in the conjugated form as E1-sulfate (Andreolini et al., 1987). Besides of humans, the excretion of E1 have been also proven in the case of wildlife and livestock (Ying et al., 2002; Yu et al., 2004; Fan et al., 2007; Feng et al., 2010). It was reported that the amount of E1 discharged to receiving water is more than 10 times comparing with E2, which means that E1, among natural estrogens, is the most important EDC in the aquatic environment (D'Ascenzo et al., 2003).

Occurrence of E1 in aquatic system

The presence of E1 in the aquatic environment was proven in many studies, among which the highest concentration of E1 in the STPs effluents was detected (Ternes et al., 1999a). Baronti et al. (2000) detected the inlet concentration of E1 of 52 ng/L, while outlet level of E1 was larger than inlet level and the average removal efficiency of E1 was about 61% (Baronti et al., 2000). The relatively large outlet level of E1 can be explained by transformation of E2 to E1 or by deconjugation of sulfate- or glucoronide- E1 (Baronti et al., 2000; Carballa et al., 2004; Servos et al., 2005). In the different study performed by Ra et al., (2011), the highest concentration of E1 among other estrogens was detected, with the degradation efficiency equal 87.8%, which is higher than found in the study mentioned above (Ra et al., 2011).

The occurrence of E1 in the surface water has been also proven, because surface water is the first media which receive estrogens mainly arising from STPs discharges (Kolpin et al., 2002; Williams et al., 2003). Ra et al., (2011) determined the concentration of E1 in the range of 3.65-69.05 ng/L, which is similar to the results obtained in the studies in China (Lei et al., 2009). In both studies the concentration of E1 in river water samples was higher comparing with the concentration of E2 (Ra et al., 2011, Lei et al., 2009), which is resulting in the easy degradation of E2 into E1 (Williams et al., 2003). The transformation of E2 to E1 under natural water conditions, occurs in a very short time, which was proven by Ternes et al. (1999b), who reported 95% of E2 degradation into E1 within 3h (Ternes et al., 1999b). The other study reported the transformation of E2 to E1 in the range time of 0.2 to 9.0 days in the river water in the presence of different amount of bacteria (Jürgens et al., 2002).

Studies of E1 bio- and photodegradation

In the literature, there are several studies about degradation of E1 in the natural environment by using microorganisms and oxidation processes. Natural estrogens are biodegraded by microorganisms, which use them for growth (Alexander, 1994). The biodegradation of E1 has been proven in agricultural soils (Colucci et al., 2001) and in the English river waters (Jürgens et al., 2002). Different study proved the possibility of E1 degradation by using nitrifying activated sludge and ammonia-oxidizing bacterium – *N. europaea* (Shi et al., 2004).

Beside biodegradation of E1, several studies have demonstrated the efficiency of UV irradiation to degrade of E1. The study performed by Liu and Liu (2004) have showed susceptibility of E1 to photolysis, because of the presence of photoactive phenolic group, which absorbs the UV radiation. They proved, that the photolysis reaction correspond with pseudo-first-order law, and suggested the breakage and oxidation of aromatic ring of E1, causing the formation of compounds with carbonyl group (Liu and Liu, 2004). The studies of E1 photolysis, in the high concentrations (3-20 mg/L), have proved the possibility of degradation under wavelength from UV and visible spectrum, although the UV radiation was found as a more effective, than UV-Vis radiation (Liu and Liu, 2004). The degradation of E1 using oxidation processes, such as O_3, H_2O_2, UV and their combination were also

studied (Sarkar et al., 2014). From all processes tested, the fastest degradation of E1 was observed during ozonation together with UV followed by O_3/H_2O_2 and almost all of E1 was degraded in 30 min (Sarkar et al., 2014). Coleman et al., have determined maximum absorbance peak of E1 in the UV area of the electromagnetic spectrum, which make it a candidate for UV photodegradation (Coleman et al., 2004). Despite, λ_{max} for E1 is known as about 283-285 nm (Lide and Bruno, 2012), its molar extinction coefficient at 281 nm is 2000 M^{-1} cm^{-1} (Chan et al., 2012), which cause the poor ability to photolysis, particularly at low environmental concentration. Additional, increasing of E1 photodegradation by combining UV with ozone has been proven (Sarkar et al., 2014).

Photodegradation rates of E1 under UV radiation and the impact of DOC on the degradation, were investigated by Atkinson et al. (2011). They determined that DOC acts as a competitive inhibitor and decreases rates of degradation during direct photolysis of E1. It means, that environmental degradation rates of estrogens can be predicted from the UV intensity reaching the surface. The studies have also showed, that the estrogenicity of estrogens (E1 and EE2) exposed to UVB didn't decrease, compared to non-UVB-exposed estrogens, which means that some of the photoproducts have also estrogenic potency (Atkinson et al., 2011). Other studies were performed by Chowdhury et al. (2010), who investigated photodegradation of E1 in aqueous solution under natural solar irradiation, using a solar simulator. They detected and studied the effects of HS and other parameters, such as solar intensity, initial concentration and pH, on the degradation of E1. The half-life of E1 was determined as 48-123 min, depending on concentration and irradiation intensity, which proves rapid degradation of E1 due to both oxidation and direct photolysis. The results of investigations showed, that the maximum E1 degradation occur at neutral pH and 8 mg/L of HA. These authors detected the major intermediate, such as benzeneacetic acid and phenylacetic acid, which were also photodegraded with time. The analysis of TOC degradation, which showed a gradual mineralization of E1, has been also performed (Chowdhury et al., 2010).

The study performed by Caupos et al. (2011) demonstrated the ability to natural photodegradation of E1 under sunlight and enhancing effect of the presence of HSs was proven. They studied the formation of photoproducts and used HPLC, UV-Visible and mass spectrometry (MS) analysis to obtain structures of photoproducts (Figure 2.)

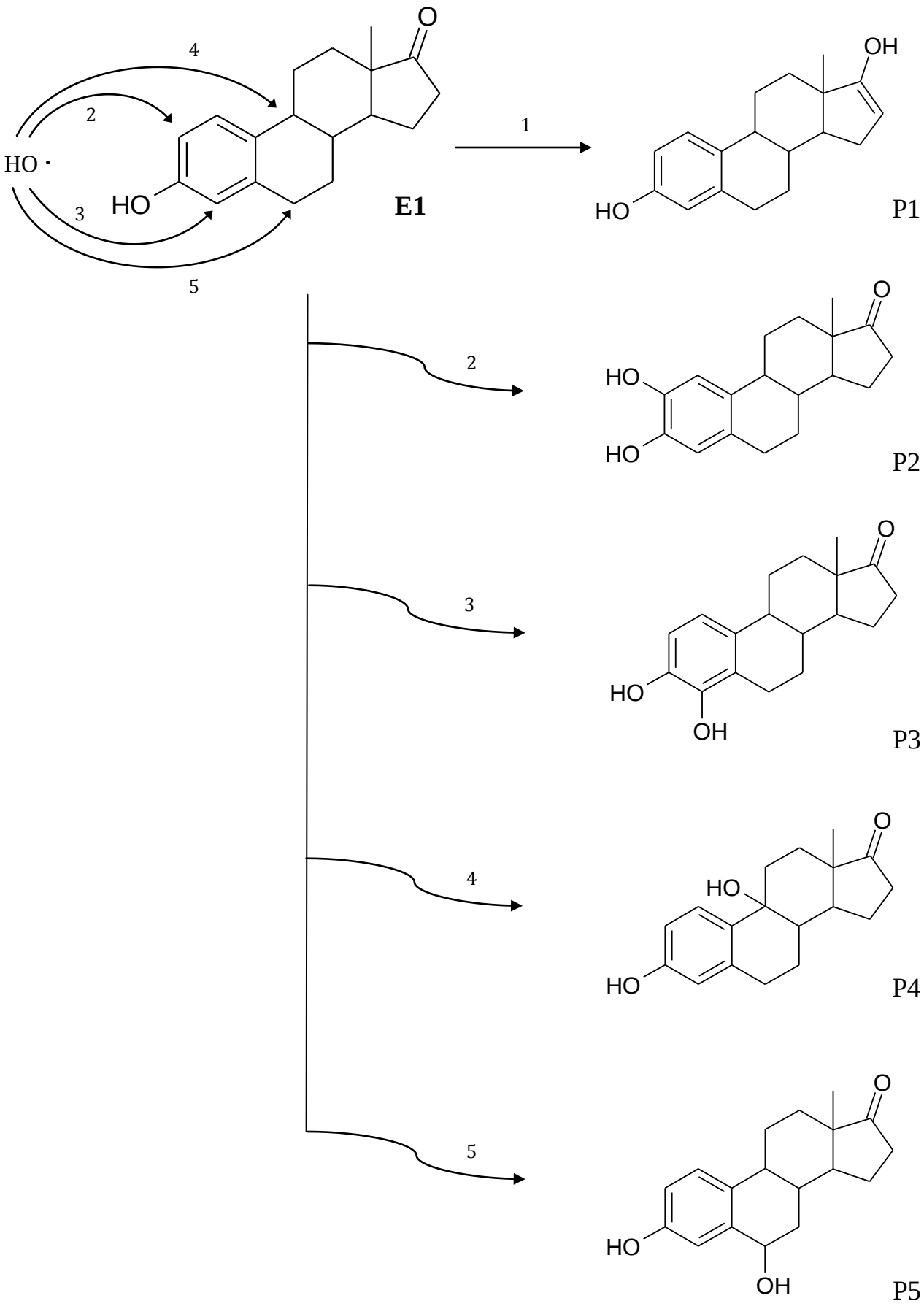


Figure 2. *Chemical structures of E1 photoproducts (based on studies of Caupos et al., 2011).*

The authors studied the photodegradation of E1 in the presence and absence of HSs and the results of HPLC-UV analysis determined the formation of only one photoproduct (P1), in the case of E1 solution without HSs. The retention time of P1 was higher than E1, but the additional analysis of GC-MS spectra showed the presence of the same major fragments in both compounds and LC-MS spectra, which were obtained in the experiment were identical. These results suggest that P1 is an isomer of E1. In addition, UV-Vis analysis was done and the identical spectra for P1 and E1 were obtained, which means that aromatic moiety have not changed. The structural changes were observed in the steroid moiety and enol structure has been proposed for P1 photoproduct. Formation of P1 has been explained by direct photolysis of E1, which was proven by the decreasing of amount of P1, when HS was added (Caupos et al., 2011). Photodegradation experiment were also performed in the presence of HSs, which resulted in the formation more photoproducts (P2, P3, P4, P5), with retention time lower than E1. Their formation were also evident in a small degree in purified water. On the basis of LC-MS analysis found that these four photoproducts were formed in the results of hydroxylation processes. In the case of E1, hydroxylation may take place in *ortho* position of the phenolic group on the aromatic ring (P2 and P3) or in the position 6 and 9 onto alicyclic ring (P4 and P5). Summing up, the irradiation of E1 caused the formation of 5 photoproducts, from which 4 products are the result of E1 hydroxylation and one compound, which appeared with the same mass as E1, is certainly an isomer of E1 (Caupos et al., 2011).

The object of this work is study kinetic of E1 degradation under simulated solar irradiation and the effect of HSs and scavengers on the photodegradation. The fractions of HA, FA and XAD-4, collected from freshwater stream, have been chosen to investigation and impact of their concentration will be studied. To understand the behavior of E1 in the aquatic environment, the study of photodegradation in water samples, containing naturally DOM, has to be carried out. Due to the difference in the content of DOM, the samples from different origins: estuarine water, fresh water and wastewater samples have been collected and used to the investigation. Few studies concern the impact of scavengers on the photodegradation of E1, therefore in this work this effect will be studied and explained, using sodium azide, as an singlet oxygen scavenger. Beside the study in purified water, samples from STPs has been also chosen to investigate the effect of sodium azide on the photodegradation.

Materials and Methods

I. Instrumentation

II. Chemicals

III. Water samples

IV. Analytical procedure

E1 solutions' preparation

Photodegradation experiments

I. Instrumentation

All samples were irradiated under simulated solar radiation using a Solarbox 1500 (Co.fo.me.gra, Italy). The irradiation device contained an arc xenon lamp (1500 W) and outdoor UV filters that limited the transmission of light with wavelengths below 290 nm. The irradiance of the lamp was 55 W/m^2 (290-400 nm), and was kept constant during all the experiments. To monitor the irradiance level and temperature, a multimeter (Co.fo.me.gra, Italy), that was equipped with a UV 290-400 nm large band sensor and a black standard temperature sensor, was used. Through an air cooled system, the device was being refrigerated. Furthermore, a parabolic reflection system guaranteed the uniformity of the irradiation inside the chamber.

Quantitative analysis of E1 was achieved by a High-Performance Liquid Chromatograph Prominence system equipped with a fluorescence detector (HPLC-FLD). This device consisted of a degasser DGU-20A5, a column oven CTO-10ASVP and a bomb LC-20AD (all from Shimadzu). For the separation, a New ACE® C18 column-PFP (5 µm, 150 mm x 4.6 mm) connected to an ACE® 5 C18 (4.6 inner diameter) guard column and an injection loop of 20 µL were used. Either the cell and column temperatures were maintained at 25°C. The mobile phase consisted of a water:acetonitrile mixture (40:60, v/v) and the flow rate was 0.8 mL/min. Before use as mobile phase, water and acetonitrile were filtered through a 0.2 µm polyamide membrane filters (Whatman).The detection was done by using a Prominence RF-20A XS fluorescence detector from Shimadzu with an excitation wavelength of 280 nm and an emission wavelength of 310 nm.

All UV-Visible spectra were obtained with a Shimadzu UV 2101 PC spectrometer in rectangular quartz cuvettes with an optical path length of 1 cm, between 200 and 600 nm. Fluorescence analyses were performed in a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon) with a Xe lamp source, using 1 cm quartz cuvettes and 5 nm bandwidths. To obtain the fluorescence emission spectra (obtained with a scan speed of 100 nm/min), the sample was excited at 280 nm and then the emission wavelengths (in the range 300-550 nm) were read.

Total organic carbon (TOC) was measured using a TOC-VCPH Analyzer, from Shimadzu.

II. Chemicals

E1 (purity >99%, E1) used in these studies was provided by Sigma-Aldrich. Ultrapure water used in the preparation of solutions was obtained from a Milli-Q Millipore system (Milli-Q plus 185). Acetonitrile (HPLC grade), used for HPLC analysis, was obtained from VWR, Prolabo.

To study the effect of OM on photodegradation of E1, three fractions of HS were used. These fractions, HA, FA and XAD-4, were extracted and isolated from a riverine water sample, collected from a freshwater stream (Poço da Cruz, Mira) that flows into the Aveiro Lagoon (Portugal, 40°39'N, 8°44'W). For the extraction and isolation of the different fractions of HS, a system of XAD resins (XAD-8 and XAD-4) in series was used. This procedure was performed and described in Santos et al. (1994) and Esteves et al. (1995). The purified fractions of HS were subsequently characterized by elemental analysis (Table 4) and solid-state ^{13}C-CPMAS NMR (Esteves et al., 2009).

Table 4. *Elemental analysis of HS fractions used (HA, FA and XAD-4); (adapted from Calisto et al., 2011).*

	Elemental analysis (%) *					
	C	H	N	S	O	C:N
HA	51.4	4.3	4.2	2.1	32.1	12.2
FA	54.0	4.8	1.9	1.5	35.2	28.4
XAD-4	49.2	4.4	2.9	1.5	40.0	17.0

** The results were corrected for humidity at 60°C and ashes at 750 °C (Calisto et al., 2011).*

The influence of a specific scavenger on the photodegradation of E1 was also studied by using sodium azide, NaN_3 ($\geq$ 99%), from Riedel-de Haën.

III. Water samples

Surface and waste water samples were collected between February and April 2014. Immediately after collection, all samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4˚C prior to use.

One surface sample, which was collected from Ria de Aveiro located in the urban city center of Aveiro, was of estuarine origin (Fonte Nova, Ria de Aveiro, Aveiro, Portugal). This sampling site is typically known to have high contents of organic matter and salinity. The other surface water sample tested was a freshwater sample from Rio Novo do Príncipe (Aveiro, Portugal), located in the rural and agricultural area of Aveiro. Two wastewater samples were also collected from one of the Aveiro's sewage treatment plants (STPs), in two different stages of the treatment – after primary treatment (STPP) and in the final effluent (STPF).

IV. Analytical procedure

E1 solutions' preparation

Due to the low solubility of E1 in water, acetonitrile was used as an auxiliary solubilizing agent. Stock solution of 1 g/L E1 was prepared by dissolving 10 mg of E1 in acetonitrile and stored at 4°C. The calibration curve of E1 was obtained by preparation of standard solutions in Milli-Q water, with concentrations: 1000, 500, 250, 100, 50 and 10 μg/L, by dilution of proper amounts of the stock solution.

To study the photodegradation of E1, a final concentration of 500 μg/L of standard solution was chosen. This work solution was prepared from the stock solution of E1 by diluting a proper amount with Milli-Q water.

The final concentration of acetonitrile did not exceed 1% (v/v) of the irradiated samples in order to avoid the influence of acetonitrile on the photodegradation rate. The pH of the irradiated solutions was not corrected and the samples were not buffered to avoid effects of the buffering agent in the photodegradation process.

Photodegradation experiments

The standard solution of E1 was transferred into quartz tubes (internal diameter of 1.8 cm and height of 20 cm), which were irradiated under simulated solar radiation in Solarbox 1500. All irradiations were performed using an irradiance of 55 W/m^2 (290-400 nm), that corresponds to 550 W/m^2 in all spectral range. For each set of experiments, 4 tubes were introduced into the SolarBox: three of them were exposed to radiation and the other one was covered with several layers of aluminum foil to protect it from light (dark control). The dark controls were kept inside the SolarBox during the same time as the irradiated solutions. In all experiments no concentration decrease of E1 was observed in the dark control experiments indicating no degradation by microbiological or thermal means, but only photo-induced. To hold the quartz tubes suspended inside the irradiation chamber, a home-made metallic holder was used, which allowed the homogeneous irradiation. From each irradiated samples and dark controls, 1 mL aliquot was collected at specific time intervals and stored at 4°C for subsequent analysis by HPLC-FLD. The degradation percentage for each set of experiments was calculated in relation with the respective dark control.

For the E1 kinetic studies in MQ water, a working solution of 500 μg/L E1 was prepared, transferred into quartz tubes (25 mL in each tube) and, in a first approach, subjected to 24 h cycles of photodegradation. However, after the first 24 h of irradiation, HPLC-FLD analysis of the irradiated samples did not show any peak of E1, which indicated the total degradation of the hormone. Therefore, aliquots were collected after 30 and 60 min and then every hour until a maximum of 10 h of irradiation. Then, the samples were analysed using HPLC-FLD.

For the study of the effect of HS on the E1 photodegradation, kinetic studies were also performed by using three fraction of HS (HA, FA and XAD-4) in a concentration of 20 mg/L each one. The solutions of 500 μg/L E1 in HA, FA and XAD-4 were prepared with Milli-Q water and subjected to irradiation during 10 h. Aliquots were collected after 30 and 60 min and after that every hour until a maximum of 10 h of irradiation (as in the kinetic study of E1 in MQ).

The experimental data of all the kinetics experiments were fitted by using GraphPad Prism 5.

The effect of concentration of HS on the E1 photodegradation was also studied. For this purpose, solutions of 500 μg/L E1 in presence of 30 mg/L and 40 mg/L HS were prepared. This experiment was carried out for each fraction of HS. The solutions were irradiated in quartz tubes during 2 hours and then quantitatively analysed by HPLC-FLD.

In order to investigate the photodegradation of E1 in environmental samples, each one of the real water samples was spiked with 500 μg/L E1, distributed into quartz tubes and irradiated during 2 h.

The experiments of E1 photodegradation in the presence of a specific scavenger - 20 mM of sodium azide (as a singlet oxygen scavenger) - were also performed in MQ water and in the STPP and STPF samples. Solutions were subjected to 2 h of irradiation and then analysed using HPLC-FLD.

Results and Discussion

i. HPLC-FLD methodology

ii. Photodegradation studies

Direct photodegradation kinetics of E1

iii. Effects of DOC on the photodegradation

Characterization of DOC

Influence of HS on the photodegradation of E1 in MQ

Influence of HS concentration on the photodegradation of E1 in MQ

Photodegradation of E1 in water samples

iv. Effect of singlet oxygen scavenger on the photodegradation of E1

i. HPLC-FLD methodology

The calibration curve was performed by using five standard solutions of E1 with concentrations ranging from 10 to 500 µg/L. These solutions were prepared by dilution of a stock solution of 1 g/L E1 in MQ water and were subjected to HPLC-FLD analysis (with the conditions described above). Peak area of the analyte was plotted as a function of the analyte concentration. The value of the coefficient of determination (R^2) of 0.9994, confirms the excellent linear response in the studied range of concentrations (Figure 3).

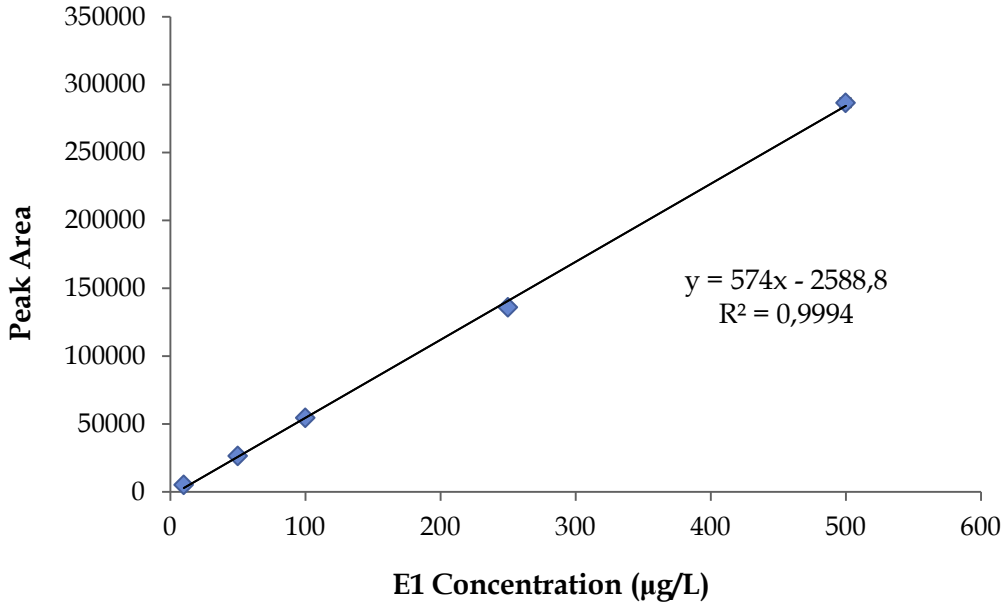


Figure 3. *Calibration curve of HPLC-FLD method. Calibration curve equation is also shown.*

The calibration curve allowed obtain a detection limit (LOD) of 17.4 µg/L, that was calculated according to equations below (Miller and Miller, 2000):

$$LOD_y = a + 3\,S_{y/x} \qquad \text{(equation 1)}$$

where,

LOD_y – is the value of LOD in the y-axis;

a – is the intersection with the y-axis;

$S_{y/x}$ – is the statistical parameter that estimates the random errors in the y axis;

and

$$S_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}} \qquad \text{(equation 2)}$$

where,

y_i – is the experimental values of y obtained for each calibration standard;

$\hat{y}_i$ – is the calculated y-values by using the calibration curve equation, corresponding to the individual x-values of standards;

n – is the number of standard solutions that were used for calibration.

The peak areas of E1 in the chromatograms of these standards were used to calculate the concentration of E1 by using the calibration curve equation. The values of concentrations obtained from calculations did not differ more than 0,1% in comparison with the real concentrations. The results, obtained in the calibration procedure, allowed performing photodegradation studies of E1.

ii. Photodegradation studies

The study of direct photodegradation of E1 was performed in ultrapure water to investigate the rate of degradation in the absence of OM.

Direct photodegradation kinetics of E1

The photodegradation of E1 was investigated in ultrapure water, irradiating the solution during 10 hours and results showed a decrease of E1 concentration with time (Figure 4). The experimental data of C/C_0 against time were fitted by non-linear regression, according to the equation:

$$C/C_0 = e^{-kt} \qquad \text{(equation 3)}$$

where C and C_0 are the concentrations of E1 exposed to light and protected from it, respectively, for different irradiation times; k is the pseudo first-order degradation rate constant (h^{-1}) and t is time (h).

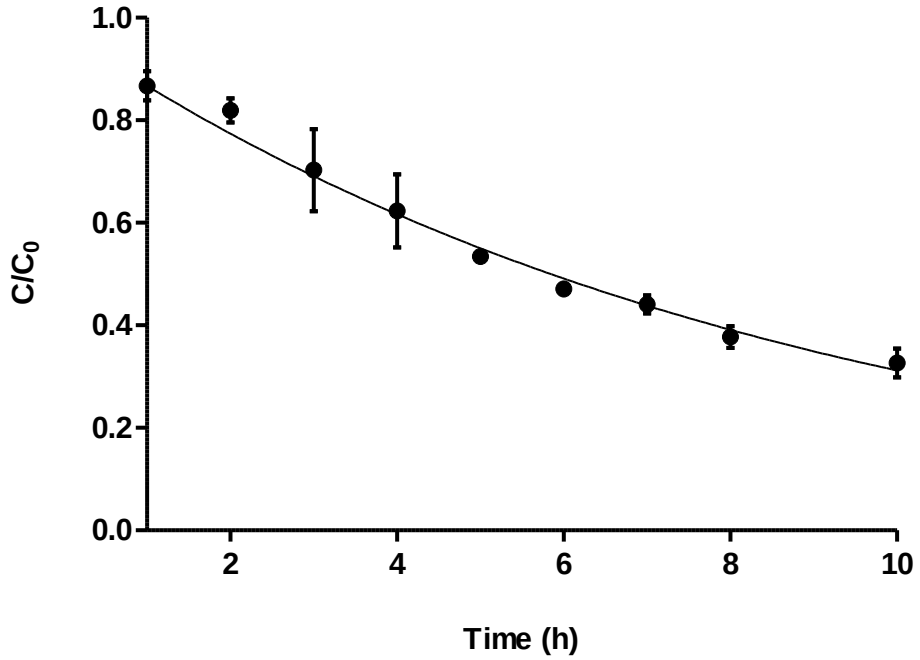


Figure 4. *Kinetics of E1 photodegradation in ultrapure water (direct photodegradation).*

To calculate the half-time of E1, the following equation was used:

$$t_{1/2} = \frac{ln2}{k} \qquad \text{(equation 4)}$$

Photodegradation of E1 followed a pseudo first-order kinetics model, which is in accordance with literature (Zhang et al., 2007; Chowdhury et al., 2010; Caupos et al., 2011), with a rate constant of 0.1137 ± 0.005 h^{-1}. The same kinetic model was obtained in the study of photodegradation of EE2 (Liu et al., 2003; Grzybowski and Szydłowski, 2014), E2 (Zhang et al., 2007; Mazellier et al., 2008), and also of psychiatric pharmaceuticals (Calisto et al., 2011). The kinetic results indicated fast degradation of E1 in MQ with a half-life time of E1 of 6 h. No degradation was observed in the tubes covered with aluminum foils (control). The rapid direct photodegradation of E1 was also proved by Caupos et al. (2011), who obtained a first order rate constant of 0.090 ± 0.006 h^{-1} and a half-life time ($t_{1/2}$) of E1 of 8 h.

iii. Effect of HS on the photodegradation

Characterization of HS

To investigate the effect of HS on the photodegradation of E1, three fractions were used – 20 mg/L HA, FA and XAD-4. Each one of them has different chemical and optical properties (Martin-Mousset et al., 1997; Sierra et al., 2005) that can change the degree of degradation. The presence of chromophoric compounds (those with aromatic structures) and fluorophores lead to UV absorbance and fluorescence emission properties, respectively. The purified fractions HA, FA and XAD-4 were characterized by Esteves et al. (2009), using solid-state ^{13}C-CPMAS NMR and results are shown in Table 5.

Table 5. *Solid-state ^{13}C-CPMAS NMR data of HS fractions used (HA, FA and XAD-4) (adapted from Calisto et al. (2011).*

^{13}C-CPMAS NMR spectra fraction (%) in the specified chemical shift range (ppm) *							
	0-60 (alkyl and methoxyl carbons)	60-90 (O-alkyl carbons)	90-108 (anomeric carbons; carbon hydrates)	108-145 (aromatic carbons)	145-160 (O-substituted aromatic carbons)	160-190 (carboxylic and ester carbons)	190-220 (carbonyl; carbons; ketones and quinones)
HA	51.3	14.4	3.2	18.6	3.4	7.3	1.8
FA	61.1	14.0	2.4	10.3	1.7	8.4	2.1
XAD-4	55.9	21.5	3.6	7.4	1.4	9.1	1.1

** NMR data represents the percentage area of the spectra fraction due to the carbons in the specified chemical shift range in ppm. Functional groups whose carbons present resonance in the specified range are given in parentheses (Calisto et al., 2011).*

HSs are complex molecules, which consist of aromatic cores, substituted with functional groups and aliphatic cores (Aleksandrova et al., 2011) with different heat resistance. The research performed by Esteves and Duarte (1999) demonstrated different thermal behavior of three fractions of HS, which resulted from the different chemical structures of each one of them. FA and XAD-4 fractions have proven to have the great content of thermally labile components that decompose at lower temperatures, while HA only decompose at higher temperatures. This is the result of the higher aromaticity of HA

in comparison with FA and XAD-4 and is in accordance with the data of ^{13}C-CPMAS NMR above (Table 5). HA was found to be the most thermo-resistant and only decomposes at temperatures higher than 300°C, which suggest a lower content of aliphatic chains and functional groups, comparing with FA and XAD-4 fractions (Esteves and Duarte, 1999).

To investigate the structural differences of three extracts of HS, TOC analysis (Table 6), UV-visible spectra (Figure 5) and fluorescence spectra (Figure 6) were performed. TOC analysis (Table 6) indicates the greater content of organic carbon in HA, comparing with FA and XAD-4 content, which is also confirmed by structural characterization of these compounds (Table 5). The results of ^{13}C-CPMAS NMR analysis show a higher prevalence of aromatic moieties in HA than in FA and XAD-4 (spectra fraction between 108-145 ppm for aromatic carbon and between 145-160 ppm for O-substituted aromatic carbon). The greater content of chromophores allows for more light absorption, which is confirmed by UV spectra (Figure 5).

Table 6. *TOC analysis of three fractions of HS – HA, FA and XAD-4.*

Sample	TOC (mg/L)	TC (mg/L)	IC (mg/L)
HA (20 mg/L)	12.09	12.09	0
FA (20 mg/L)	11.85	11.85	0
XAD-4 (20 mg/L)	10.81	10.81	0

The UV spectra of E1 (C=10 mg/L) and three extracts of HS were performed in the range 200–600 nm and show the decrease of absorbance values from 200 to 600 nm for all HS isolates (Figure 5). The differences in absorbance intensities are most likely caused by the difference in chromophore concentrations. Through the investigated range of absorbance, the highest values were obtained for HA, while the lowest for XAD-4, which indicates the high sensitivity of HA to radiation. The same results were obtained by Esteves et al. (2009) in the study of HSs from different origins.

To study the effect of HS on the photodegradation of E1, the absorbance values for the wavelength of 295 nm were measured in solutions of the analyte in absence of HS (MQ water) and in presence of HA, FA and XAD-4. The results showed the increase of absorbance values in the order:

$$MQ\ water\ (0{,}069) < XAD-4\ (0{,}215) < FA\ (0{,}271) < HA\ (0{,}448),$$

which can suggest that the rate of fotodegradation of E1 in presence of HS could decrease in the same order.

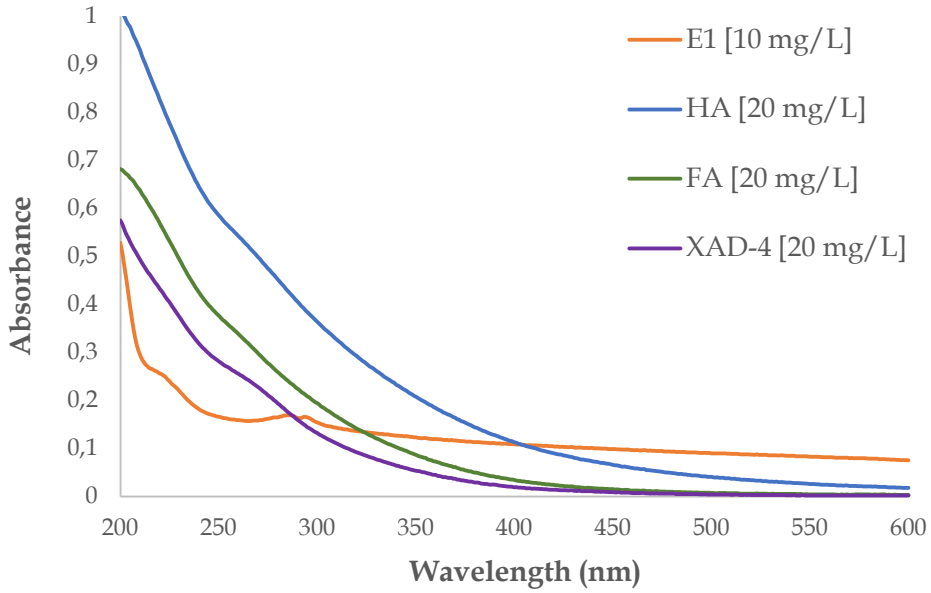


Figure 5. *UV spectra of E1 and three fractions of HS.*

The fluorescence emission spectra (Figure 6) were performed for 20 mg/L isolates of HS in the range of 300–550 nm with an excitation at 280 nm. The knowledge on HS fluorescence properties have been obtained from a single-scan fluorescence data. Single-scan fluorescence emission spectra of HS present a single and broad band whose maximum emission (λ_{max}) moves depending on the adopted excitation wavelength (λ_{ex}) (Senesi, 1990; Sierra et al., 2000). The fluorescence study of HSs gives information relating to structure, conformation, functional groups and heterogeneity (Mobed et al., 1996). It was proven before that HS, irrespective of its origin, show characteristic fluorescence spectra, which are caused by the presence of aromatic fluorophores involving electron-donating functional groups (Datta et al., 1971).

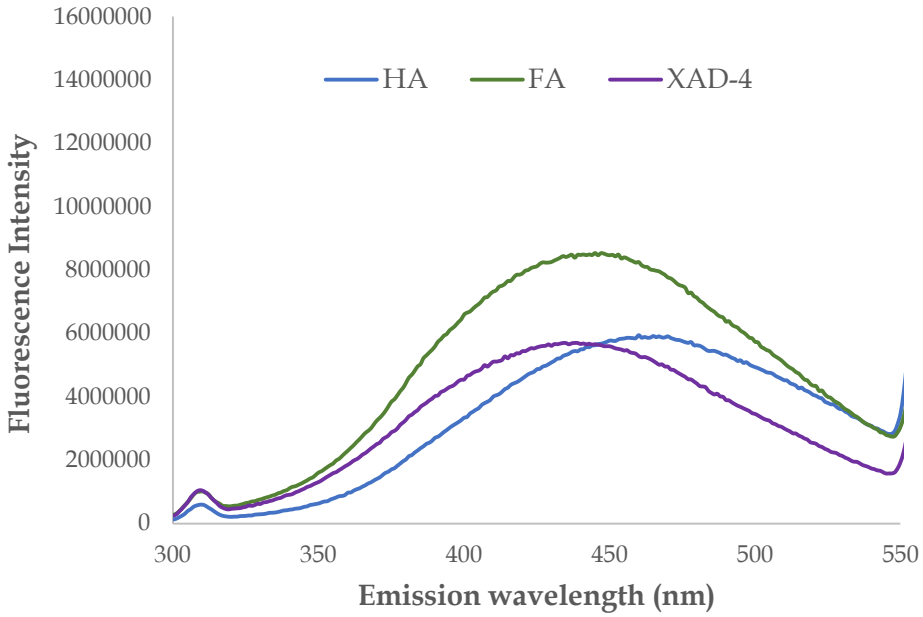


Figure 6. *The fluorescence emission spectra of three fractions of HS.*

The fluorescence emission spectra (Figure 6) of the different HS fractions, shows the same shape with a single broad band, but different intensity for each HS studied. It has been studied that high fluorescence intensity is related to low mass components and low condensation of aromatic rings (Senesi et al., 1991; Belin et al., 1993; Sierra et al., 2000). For a λ_{ex} = 280 nm, FA fluoresces with higher intensity and shorter wavelength than HA from the same origin, which is in accordance with literature (Belin et al., 1993; Sierra et al., 2000) and can be related to a higher degree of aromatic moieties and molar mass of the last one. These results are confirmed with the UV-spectra, which showed the highest values of absorbance for HA, which means the greater amount of aromatic moieties.

The fluorescence emission spectra of HA and XAD-4 represent the same shape, with similar values of the maximum fluorescence intensities. However, a shift of the HA spectrum from shorter to longer wavelengths has been observed. The peak emission wavelength of HA shifts towards longer wavelengths with increased molecular size and aromatic content, which is in agreement with the literature (Chen et al., 2003). HA is known to be rich in aromatic moieties and its lower fluorescence intensity can be partially caused by its highly substituted aromatic structures and its inter- and intra-molecular

bonding in humic macromolecules (Miano et al., 1988). In the literature these effects was explained, as a result of greater proximity of aromatic chromophores, which increase the probability of deactivation of excited states by inner quenching in organic molecules (Senesi, 1990).

Influence of HS on the photodegradation of E1

The studies of photodegradation of E1 were performed in the presence of the three HS extracts – HA, FA and XAD-4. All the solutions contained 20 mg/L of HS were irradiated during 10 h. The experimental results were fitted by non-linear regression, according to the equation 3, in the same way as before for E1 photodegradation in ultrapure water (*cf.* section *ii*). The kinetic curves of E1 photodegradation in presence of different HS extracts are presented in Figure 7. Also, kinetic curve in MQ water (absence of HS) is presented for comparison.

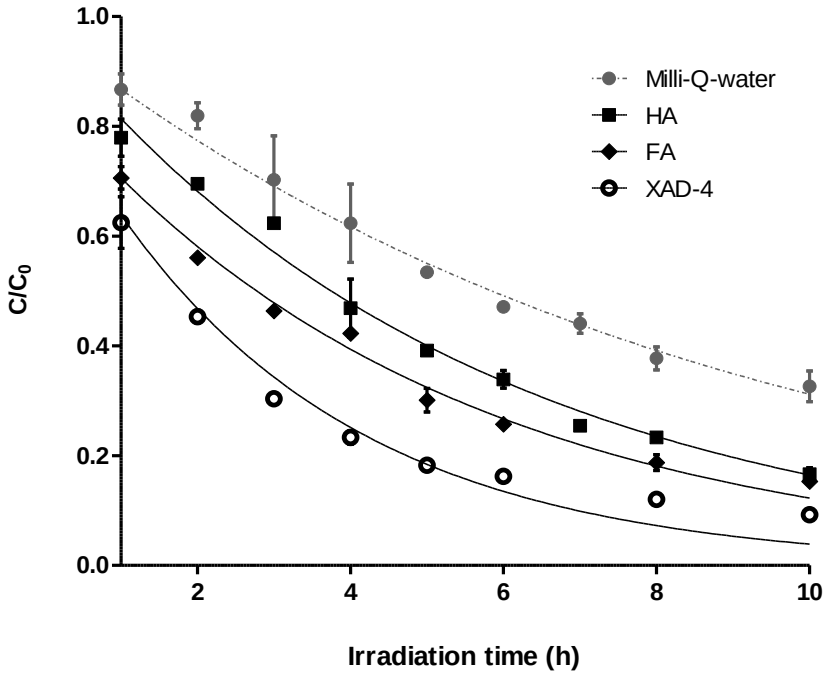


Figure 7. *Kinetics of E1 photodegradation in absence and presence of HS fractions.*

In the presence of HS, the photodegradation of E1 can happen as a direct phototransformation by absorption of light or can be induced by the presence of HS. The obtained data for the variation of E1 concentration along irradiation time were fitted to a pseudo first-order kinetics model and shows the significant impact of each of HS extracts studied to photodegradation of E1 (Figure 7). Addition of HA (C=20 mg/L) caused an acceleration of the rate of E1 photodegradation after 2 h of irradiation, being almost twice compared with the pure aqueous E1 solution (18% degradation of E1 in MQ and 30% degradation in HA). In the case of the other HS fractions studied, the impact was even greater and the photodegradation of E1 increased to 44% and 55% in FA and XAD-4, respectively (after 2 hours of irradiation).

Therefore, each one of the three HS isolates caused an increase in the degree of E1 degradation compared to MQ water. The results of E1 photodegradation (%) after 6 hours of irradiation were as follows:

$$HA\ (66\%) < FA\ (74\%) < XAD-4\ (84\%)$$

These results showed that XAD-4 is the most efficient to photoinduce the degradation of E1, which is in agreement with the properties of these three HS extracts (*cf.* section *iii*). Their photoinductive activity depends on the absorbance and fluorescence efficiency. Each of the HS extracts studied absorb the radiation due to the presence of chromophores' moieties. Considering the UV spectra (Figure 6), a low value of absorbance indicates the weak ability to absorb radiation, which causes that a significant amount of light can be available for E1, which can result in an increase of its degradation. The strongest photoinductive properties were obtained for XAD-4, which resulted in an E1 half-life time of about 2 h. Similar results were accomplished by Caupos et al. (2011), who demonstrated the increasing of the percentage of E1 photodegradation in presence of different isolates of FA.

In the absence of HS, a first order rate constant of 0.1137 h^{-1} was obtained, which corresponds to the direct phototransformation of E1. The value of the rate constant in presence of HS can be calculated, assuming that they do not have any photosensitizing properties, acting only as an inner filter. The calculations of the initial fraction of light, absorbed by E1 alone and in the presence of different HS extracts, were performed at 280 nm, using the equation below:

$$\left(\frac{I_a}{I_0}\right)_{E1/_m} = \left(\frac{I_a}{I_0}\right)_m \times \frac{A_{E1}}{A_m} \qquad \text{(equation 5)}$$

where,

$\left(\frac{I_a}{I_0}\right)_{E1/_m}$ - is the fraction of light that was absorbed by E1 in the presence of HS at 280 nm;

$\left(\frac{I_a}{I_0}\right)_m$ – is the fraction of light absorbed by the mixture (E1 + HS) at 280 nm;

A_{E1} – is the value of absorbance of E1 at 280 nm (constant due to the constant value of the concentration);

A_m – is the absorbance value of the mixture (E1 + HS) at 280 nm.

Since the intensity of light absorbed depends on the absorbance value and the intensity of incident light; the ratio $\frac{I_a}{I_0}$ can be calculated, by using the following equation:

$$I_a = I_0(1 - 10^{-A}) \qquad \text{(equation 6)}$$

In presence of HS, the radiation emitted by the lamp is mainly absorbed by HS extracts. As it was mentioned before, it is possible to calculate the first order rate constant of E1 degradation in the presence of HS acting only as an inner filter (k_{cal}), using the following equation:

$$k_{cal} = k_{E1} \times \frac{\left(\frac{I_a}{I_0}\right)_{E1/_m}}{\left(\frac{I_a}{I_0}\right)_{E1}} \qquad \text{(equation 7)}$$

where: $\left(\frac{I_a}{I_0}\right)_{E1/_m}$ is the fraction of light that is absorbed by E1 in the mixture (E1 + HS); $\left(\frac{I_a}{I_0}\right)_{E1}$ represents the fraction of light absorbed by E1 alone (in ultrapure water) and k_{E1} is the first order rate constant of E1 direct photolysis (in the absence of HS).

Combination of equations (5), (6) and (7) lead to calculate first order rate constant of E1 photodegradation in presence of HS fractions, as follows:

$$k_{cal} = k_{E1} \times \frac{\left(1-10^{-A}\right)_m}{\left(1-10^{-A}\right)_{E1}} \times \frac{A_{E1}}{A_m} \qquad \text{(equation 8)}$$

The contribution to photodegradation of each one of the three different isolates of HS was also studied and calculated using the equation 9:

$$contribution\ of\ HS\ [\%] = \left(\frac{k_{meas} - k_{cal}}{k_{meas}}\right) \times 100 \quad \text{(equation 9)}$$

The results obtained in different solutions are summarized in Table 7 showing the photosensitizing effect of the presence of HS in the photodegradation of E1. The half-life time of E1 in the presence of each HS isolate was lower than the one obtained in ultrapure water ($t_{1/2}$ = 6 h).

Table 7. *Apparent first order rate constants, coefficient of degradation (R^2), contribution of three different HS isolates to degradation and half-lives ($t_{1/2}$) obtained.*

Type of water	k_{meas} **(h^{-1})**	R^2	k_{cal} **(h^{-1})**	**Contribution of HS (%)**	$t_{½}$ **(h)**
MilliQ water	0.1137 ± 0.005	0.9889			6.10
HA (20 mg/L)	0.1774 ± 0.007	0.9910	0.0767	56.75	3.91
FA (20 mg/L)	0.1943 ± 0.008	0.9919	0.0915	52.92	3.57
XAD-4 (20 mg/L)	0.3109 ± 0.023	0.9800	0.0970	68.80	2.23

The results show how important is the presence of HS on the phototransformation of E1. The type of HS extract has also some effect on the rate of E1 degradation and the higher participation of XAD-4 (about 69%) in the photodegradation of E1 has been proven, which resulted in a considerably lower half-life time. It means that about 69% of the overall E1 degradation is caused by photosensitized reactions. Similar results, but with lower contribution of HS, were obtained for HA and FA extracts (about 57% for HA and about 53% for FA).

The effect of HS on the photodegradation of E1 in the present work is in accordance with the results that were obtained by Caupos et al. (2011), where a faster degradation of E1 in the presence of HS was also observed. The differences in the obtained

values can be attributed to differences in the experimental conditions. Caupos et al. (2011) used a solar simulator equipped in a Xe lamp with the light power of 250 W/m^2 and obtained a rate constant of 0.09 h^{-1}. In the present work the Xe lamp was also used but the irradiance of the lamp was lower (55 W/m^2). The differences in the rate of the photodegradation may be also explained by the different initial concentration of hormone: standard solutions of 0.1-1.0 μM were used by Caupos et al. (2011). The effect of the initial concentration of E1 on its photodegradation was already studied and the tendency of a decrease of the rate of photodegradation with the increase of the initial concentration of estrogens was proven (Liu and Liu, 2004; Chowdhury et al., 2010).

Influence of HS concentration on the photodegradation of E1

The influence of the concentration of HS extracts on the photodegradation of E1 was also studied. The experiments were carried out for three HS concentrations - 20, 30 and 40 mg/L - for 2 hours of irradiation, while the E1 initial concentration was maintained at 500 μg/L. Results are shown in Figure 8.

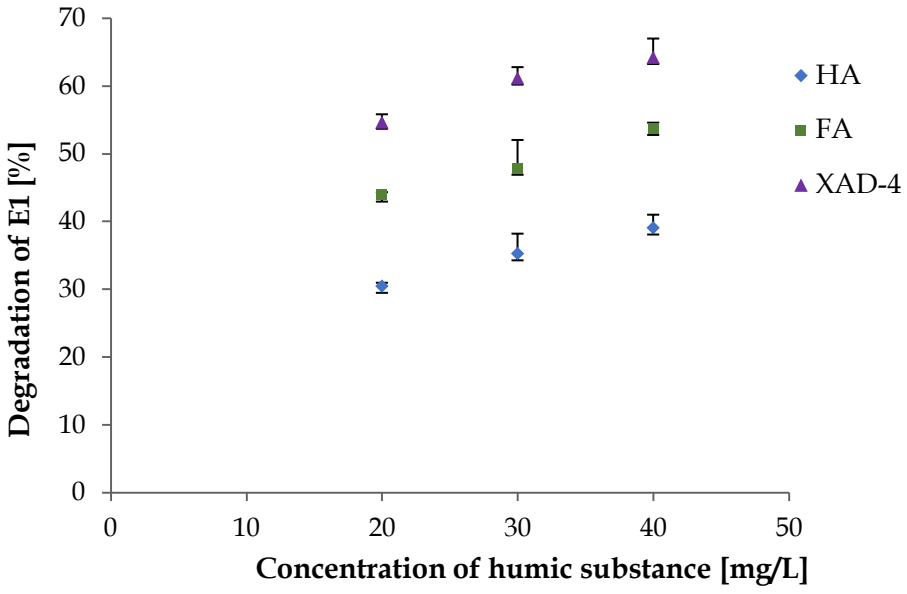


Figure 8. *The effect of HS concentration on the photodegradation of E1.*

The results show an increasing degradation of E1 with increasing HS concentrations. The same dependence was observed for each one of the three HS isolates studied and the increase of E1 concentration was similar in all of them. Degradation of E1 increased with the increase of HS concentration in the ranges 30-40%, 44-54% and 55-65%, for HA, FA and XAD-4, respectively. Similar results were obtained by Chowdhury et al. (2010), who studied the degradation of E1 in the presence of HA, in the concentration range of 0-10 mg/L, for 30 min of irradiation. These authors (Chowdhury et al., 2010) demonstrated that the degradation of E1 is enhanced with increasing HA concentration up to 8 mg/L. Same authors (Chowdhury et al., 2010) pointed out that degradation efficiency is reduced at higher concentrations of HA, due to the scavenging of reactive oxygen species. The effect of HS concentration was also studied in the case of E2 photodegradation by Leech et al. (2009), and the acceleration of degradation with increasing concentrations of HS until a particular value of concentration was proven, as well. Addition of low concentrations of HA significantly increased the photodegradation rate of E2, but further increase in degradation with increasing HA concentrations was showed to be not significant (Leech et al., 2009).

Photodegradation of E1 in water samples

Photodegradation experiments were also performed in water samples of different origins, which contain naturally existing organic matter (OM) possibly resulting in indirect photodegradation of E1. In the present work, the rate of E1 photodegradation in real water samples was investigated and compared with the results obtained in ultrapure water. The experiment was performed in four different types of water, which have different contents of OM and salinity: estuarine water (Fonte Nova, Ria de Aveiro), freshwater (Rio Novo do Príncipe) and 2 samples from a STP – STPP and STPF.

Water samples were spiked with the same concentration of E1 (C=500μg/L) as before and subjected to 2h of irradiation. The results of the photodegradation (%) of E1 in real water samples are shown in Figure 9. The percentage degradation of E1 in MQ water is also presented for comparison.

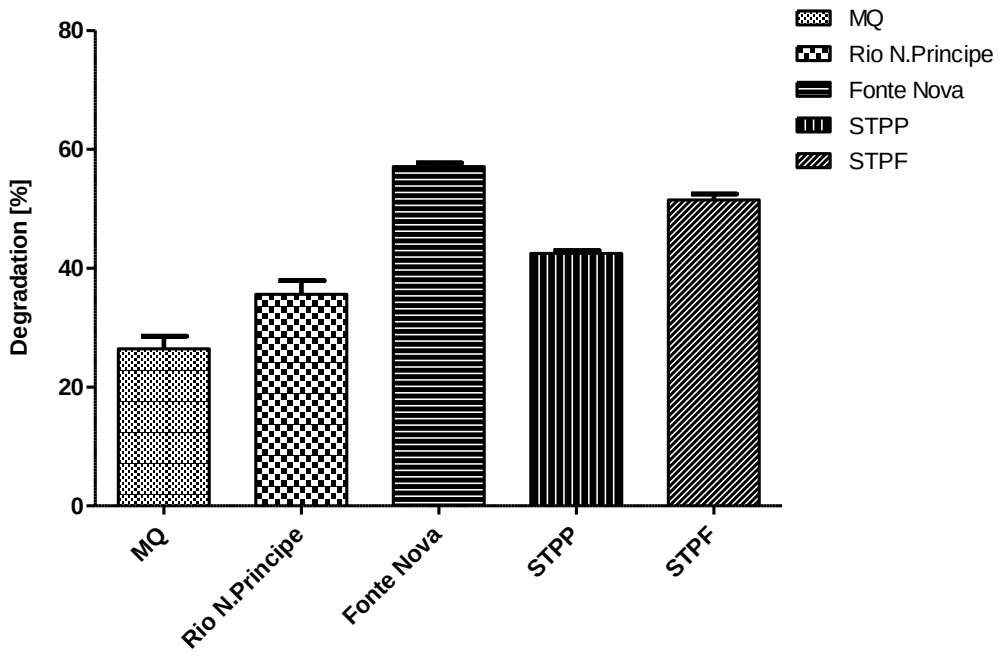


Figure 9. *E1 photodegradation (%) after 2h of irradiation in ultrapure water (MQ), freshwater (Rio N. Principe), estuarine water (Fonte Nova), primary effluent of STP (STPP) and final effluent of STP (STPF).*

The results showed that the lowest photodegradation of E1 was obtained in ultrapure water (26.4% after 2 h of irradiation). Higher photodegradation was obtained for the real water samples, increasing as follows: freshwater (35.6%) < STPP (42.5%) < STPF (51.5%) < estuarine water (57.1%). Trying to explain the different results of E1 photodegradation in real water samples, TOC analysis (Table 8), UV-visible spectra (Figure 10) and fluorescence spectra (Figure 11) were performed.

Table 8. *TOC analysis of the four types of water used.*

Sample	TOC (mg/L)	TC (mg/L)	IC (mg/L)
STPP	48.6	140.8	92.2
STPF	45.2	124.2	79.0
Fonte Nova	16.7	44.9	28.1
Rio N. Príncipe	4.8	11.7	6.8

UV-visible spectrometry (Figure 10) show the same trend for all water samples studied: decreasing of absorbance toward longer wavelengths, which corresponds with the characterization results of HS from the different origin (Esteves et al., 2009). Higher absorbance was obtained for the waste water samples, compared to surface water samples, which can be explained by higher content of organic matter (Table 8). The presence of OM increases the absorbance, mainly at short wavelengths, which can indicate a higher aromatic content (Esteves et al., 2009). The absorbance intensity was obtained as follow:

freshwater < estuarine water < STPF < STPP,

which is in accordance in increase of TOC content in all samples studied (Table 8).

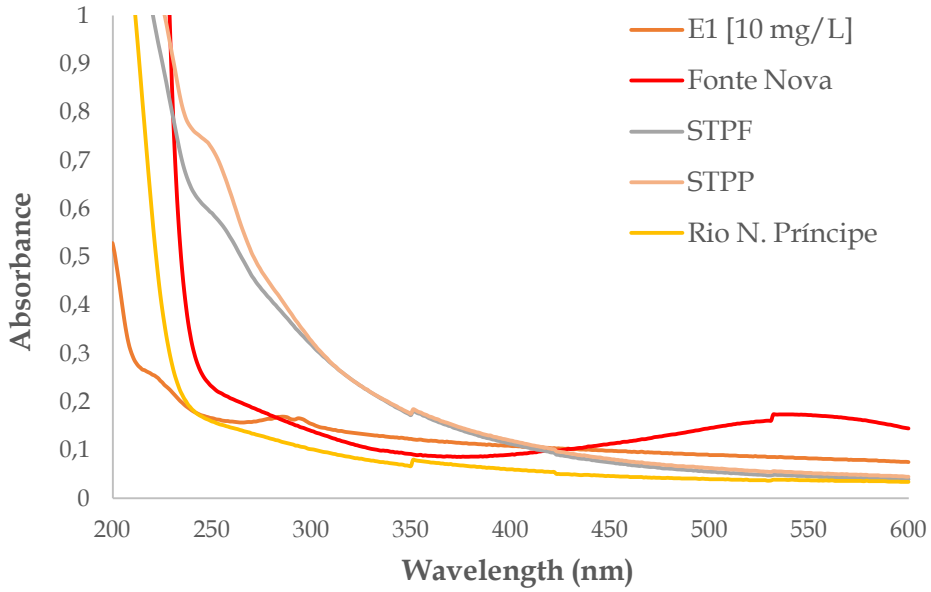


Figure 10. *UV-visible spectra of E1 solution (C=500µg/L) and four types of water samples: freshwater (Rio N. Principe), estuarine water (Fonte Nova), STPP and STPF.*

Differences in the results obtained can be explained by the different origins of the samples. The absorbance intensity of STPP was higher, compared with STPF, which suggest removal of compounds capable of absorbing light (chromophores) during sewage treatment. The surface water samples from Ria de Aveiro are known to have high levels of salinity, which result in a decrease of OM contents (Esteves et al., 1995) and is confirmed by the low absorbance (Figure 10) and fluorescence intensity (Figures 11).

In what concerns TOC analysis, in the case of STPs samples, significantly higher levels of TOC were obtained: more than ten times comparing with the fresh water sample (Table 8). The spectroscopic and fluorescence characterization shows higher chromophores' and fluorophores' content in STPs, than in surface water samples (Figures 10 and 11). The STPF water contained a low level of TOC, which resulted in a lower absorbance intensity comparing with STPP.

Overall, the different results obtained for STPP and STPF may be attributed to STP treatment stages, which include primary treatment (pre-treatment and primary decantation) and secondary treatment (biological treatment and secondary decantation).

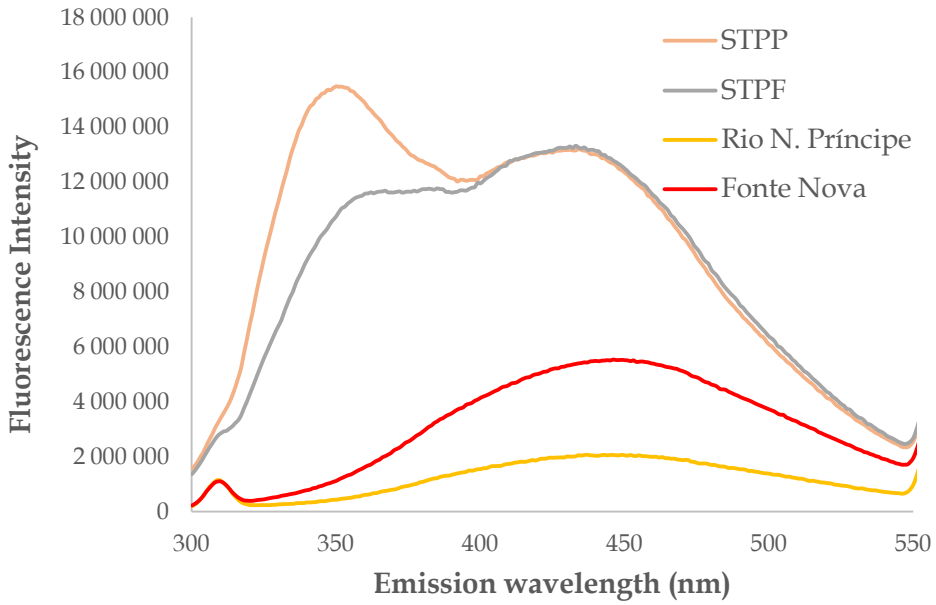


Figure 11. *Fluorescence emission spectra of four types of water samples: freshwater (Rio N. Principe), estuarine water (Fonte Nova), STPP and STPF.*

UV-visible spectroscopy is well known in the characterization of natural OM (Weishaar et al., 2003; Fuentes et al., 2006), but the fluorescence of DOC is a feature, which can give useful information about its composition (Peuravuori et al., 2002; Fuentes et al., 2006; Otero et al., 2007) and origin of HSs (Sierra et al., 2005). HS is a complex and heterogeneous mixture, in which only highly unsaturated and aromatic molecules fluoresce

(Senesi, 1990) and constitute only a fraction of all the HS components. Therefore fluorescence bands show the sum of different types of fluorophores, contained in the complex structure of HSs. Fluorescence characterization allow the observation that the origin of water samples (origin of HS also) has an effect on emission fluorescence spectra.

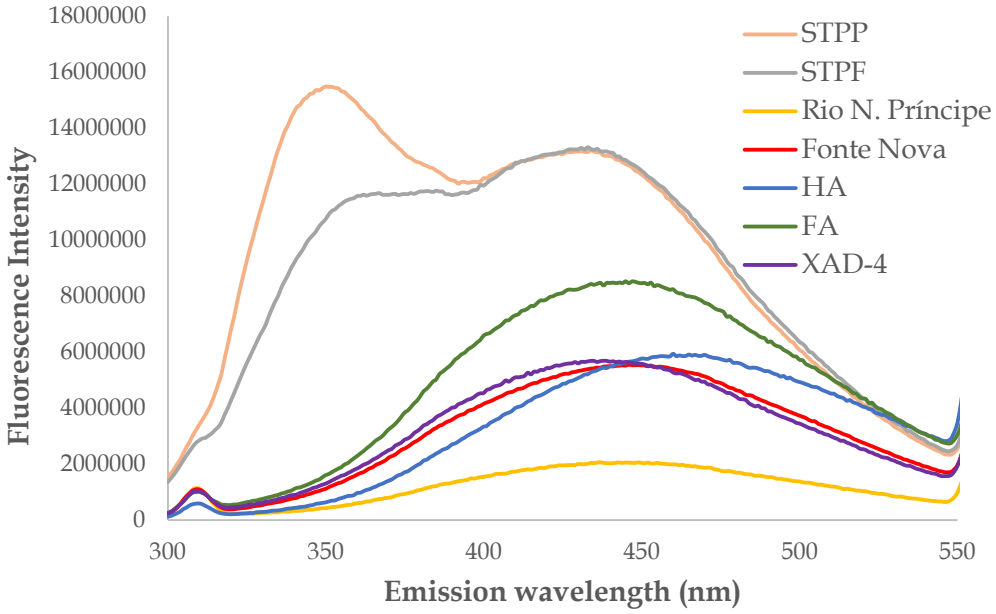


Figure 12. *Fluorescence emission spectra of all water samples: freshwater (Rio N. Principe), estuarine water (Fonte Nova), STPP and STPF and HS fractions (HA, FA, XAD-4) to comparison.*

Fluorescence emission spectra of DOM in natural water samples normally shows a broad peak, which is common for spectra of complex natural organic substances (Lombardi and Jardim, 1999 and McKnight et al., 2001). Figure 12 allows the comparison between the fluorescence spectra obtained for all the real water samples and the spectra of the HS fractions studied. The fluorescence spectra obtained for fresh water and estuarine water is similar to the fluorescence spectra of XAD-4 fraction, which suggest the predominance of this fraction on the surface water samples. The higher fluorescence intensity obtained for estuarine water corresponds to the higher fluorophores' content, comparing with fresh water (Figure 12). In the case of samples from STPs, the fluorescence spectra are similar to each other and two fluorescence bands are shown. The wide band in the wavelength range of 400-550 nm can correspond to the presence of HS fractions, mentioned in this work, predominantly FA. The identical fluorescence intensity

provides the same content of HS in both STP samples studied. The fluorescence band in the wavelength range of 300-400 nm suggest the presence of different type of fluorophore, that were not studied in this work. The lower fluorescence intensity obtained for STPF corresponds to a lower content of molecules fluorescing in that wavelength, which may be caused by their removal during sewage treatment.

iv. Effect of singlet oxygen scavenger on the photodegradation of E1

The impact of singlet oxygen scavenger on the photodegradation of E1 was studied in the samples containing E1 (C=500µg/L) and 20 mM of NaN_3 after 2h of irradiation. Experiments have been performed in ultrapure water and in wastewater samples (STPP and STPF), to study the effect of the simultaneous presence of azide ions (N_3^-) and HS on the rate of E1 photodegradation. The results of the photodegradation (%) of E1 in the absence and presence of the scavenger are shown in the Figure 13.

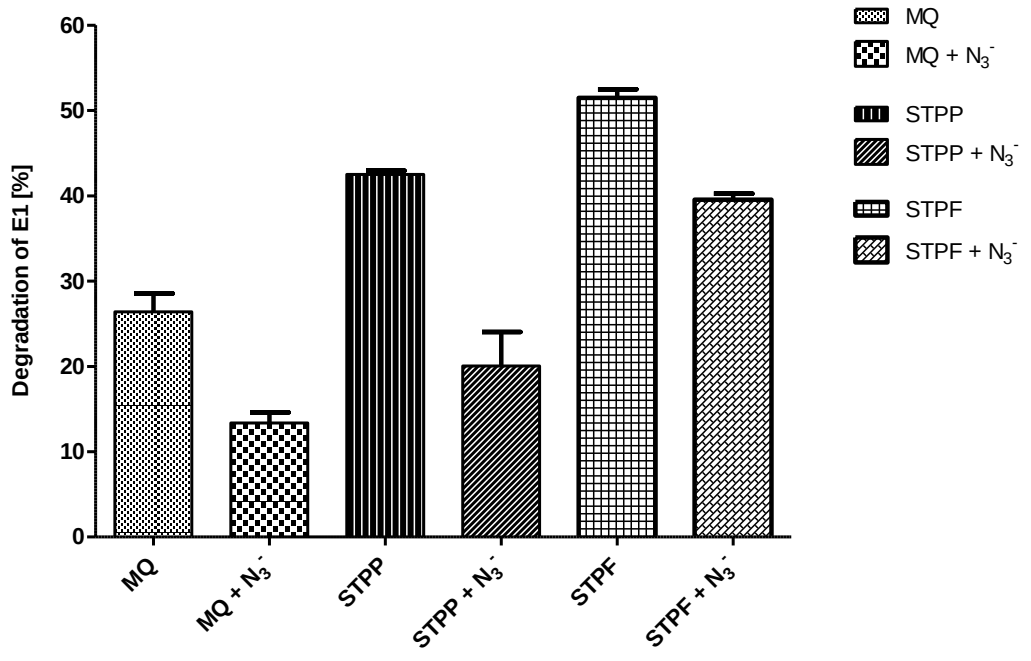


Figure 13. *The effect of NaN_3 on the photodegradation of E1 after 2h of irradiation.*

The experiment proved a significant impact of azide ions, resulting in the decrease of the rate of E1 degradation in each samples studied. The significant effect was obtained in ultrapure water, where the degradation of E1 decreased 50% in the presence of N_3^- after 2 h of irradiation (the photodegradation of E1 was 26% in MQ and 13% in MQ with N_3^-). In the case of STPP sample, the rate of E1 photodegradation decreased from 42% in the absence of scavenger to 20% in the presence of N_3^-. However in the STPF water the percentage of E1 photodegradation has changed slightly: from 51% to 40% in the absence and presence of scavenger, respectively. The difference can be explain by different content of OM.

The main reactive species responsible for the degradation of E1 in the presence of OM are hydroxyl ($HO^{\cdot}$) and oxygen radicals ($^1O_2$) (Caupos et al., 2011). The results obtained can be explained by the presence of N_3^-, acting as $^1O_2$ scavenger, inhibiting the participation of the radical in the photodegradation of E1:

$$N_3^- + {}^1O_2 \rightarrow N_3^{\cdot} + O_2 \qquad (13)$$

It was proven that NaN_3 acts mainly as a physical scavenger of $^1O_2$, but it can also react with $HO^{\cdot}$, forming the azidyl radical (N_3^-) (Bottu, 1989; Halliwell and Gutteridge, 1990). The participation of other reactive species (excited triplet state, peroxyl radicals or solvated electrons) cannot be excluded since scavengers are not completely selective (Zepp et al., 1985, 1987; Canonica et al., 1995).

The results obtained in present work are in agreement with other researchers, who studied the impact of NaN_3 on the photodegradation of E1 (Caupos et al., 2011). The results of Caupos et al. (2011) showed also a decrease of E1 photodegradation with the addition of NaN_3.

Conclusions

Conclusions

In this work, besides the study of the E1 photodegradation in presence of different fractions of HS and the impact of HS concentration, experiments were also performed in natural waters from different origins. To better understand the effect of reactive species on the rate of photodegradation, the study of the addition of sodium azide, as a singlet oxygen scavenger, was performed.

The results and conclusions from the experimental work are:

- ✓ The experiments demonstrated the possibility of E1 photodegradation under simulated sunlight, with a half-life of 6 h in purified water.
- ✓ The presence of HSs caused an enhancement of the photodegradation of E1 in all fractions tested, from which XAD-4 was the most efficient (half-life of 2 h).
- ✓ The study of E1 photodegradation in the presence of different concentrations of HS (20-40 mg/L) proved the acceleration of the E1 photodegradation with increasing HS concentrations.
- ✓ The structural differences between three fractions of HS have been proven by using spectroscopy, fluorescence and TOC analysis, which had an effect on the photodegradation of E1.
- ✓ The photodegradation of E1 in real water samples was faster comparing with ultrapure water.
- ✓ The spectroscopy, fluorescence and TOC analysis of the real water samples demonstrated different content of OM, which resulted in differences in the rate of E1 photodegradation. The impact of salinity on the photodegradation, suggested in the literature, was also suggested by the results.
- ✓ The effect of a singlet oxygen scavenger on the photodegradation of E1 was studied using NaN_3 and a 50% decrease on degradation in ultrapure water, after 2 h of irradiation, was obtained, meaning that, possibly, oxygen radicals are scavenged, not participating in the photodegradation mechanism.

- ✓ To examine the effect of both the OM and the scavenger on the photodegradation of E1, the experiment with addition of NaN_3 was performed in wastewater samples. The decrease in the E1 photodegradation in both wastewater samples was observed, but the effect was more pronounced in the case of STPP.

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